Jobst Winter

Immunological causes of bronchiolitis obliterans after lung transplantation

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Stellingen behorende bij het proefschrift

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Groningen, 16 november 1994

1 Bronchiolitis obliterans in getransplanteerde longen is niet synoniem aan chronische pulmonale uitstoting, en daarom dienen deze termen niet langer door elkaar gebruikt te worden.

2 Door de enorme belangstelling voor CMV-infecties in getransplanteerde organen worden virale luchtweginfecties onderschat als oorzaak van bronchiolitis obliterans in getransplanteerde longen.

3 Het lokale afweersysteem in de getransplanteerde long wordt door een lokale uitstotingsreactie vernietigd.

4 Door de aantasting van het lokale afweersysteem in de getransplanteerde long kan een luchtweginfectie een heftige ontstekingsreactie veroorzaken in de kleine luchtwegen die vervolgens aanleiding geeft tot het ontstaan van bronchiolitis obliterans.

5 Zonder ontsteking is er geen leven, met een inadequaat afweersysteem valt niet te leven.

6 Zolang onze kennis over de groeimogelijkheden van een volwassen long dysmatuur blijft, zal transplantatie van twee volwassen longkwabben bij kinderen met cystische fibrose (taai slijmziekte) in de kinderschoenen blijven staan.

7 Bij transplantatiepatiënten met langoverlevende organen dient bij algehele-malaiseklachten niet alleen gedacht te worden aan uitstoting of CMV-infectie, maar ook aan een relatieve bijnierinsufficiëntie ten gevolge van chronisch corticosteroïden gebruik.

8 Over vijftig jaar zullen er geen mens-naar-mens orgaantransplantaties meer worden uitgevoerd.

9 In tegenstelling tot wat veel pulmonologen èn cardiologen denken, hoort de rechter ventrikel bij de cardiologie.

10 Zolang de processen die als reactie op ballondilatatie (PTCA) van een coronair-arterie ontstaan niet worden begrepen en niet worden voorkomen, zal de chirurgische bypass van de coronair-arterie de superieure vorm van myocardrevascularisatie blijven.

11 De Nederlandse huisarts behandelt geen ziekten maar klachten

12 De consultduur van de huisarts is omgekeerd evenredig met zijn inktgebruik.
(*Johan Winter, huisarts*)

13 Om de samenwerking tussen de eerste-, en tweedelijns geneeskunde te verbeteren dient elke medisch-specialist-in-opleiding een stage van minstens 3 maanden in een huisartsenpraktijk te volgen.

14 Het verschil in omvang tussen een juridisch en een medisch proefschrift wordt vooral veroorzaakt door het feit dat juristen met zoveel mogelijk woorden zo weinig mogelijk trachten te zeggen en dat medici daarentegen het omgekeerde proberen.

15 Het cellentekort in gevangenissen en de wachttijden in ziekenhuizen moeten niet alleen bestreden worden door te voorzien in meer cellen en meer artsen, maar vooral ook door het maatschappelijk en lichamelijk normbesef van mensen te verhogen.
(*Jaap Winter, advocaat, vrij naar Socrates in Plato's Georgias*)

16 In de wetenschappelijke jacht naar $p < 0.05$ verworden de gebruikte statis*tics* vaak tot statis*tricks*.

17 Elke wetenschappelijke vraagstelling die niet als inzet van een weddenschap kan dienen is onwetenschappelijk en dient niet gesubsidieerd te worden.

18 Op de basisschool leer je basissen.
(*Thom Winter, 2 jaar, personal communication)*

19 De uitspraak "promoveren is maar betrekkelijk" is onzinnig als niet wordt vermeld in relatie tot wat.

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Immunological causes of bronchiolitis obliterans after lung transplantation

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ter verkrijging van het doctoraat in de Geneeskunde
aan de Rijksuniversiteit Groningen
op gezag vande Rector Magnificus Dr. F. van der Woude
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des namiddags te 4.00 uur

door

Jobst Berend Winter
geboren op 24 november 1961 te Groningen

Promotor
Prof. Dr. Ch.R.H. Wildevuur

Referenten
Dr. Jm. Prop
Dr. A.S.H. Gouw

Aan Papa en Mama, voor wat jullie hebben gegeven
Aan Darinka, voor wat je samen met me deelt
Aan Thom en Joyce, voor wat jullie nemen èn geven

Promotiecommissie

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"How often have I said to you that when you have eliminated the impossible, what ever remains, however improbable, must be the truth"

Sherlock Holmes, *The Sign of Four*

They did not want to look on the naked face of luck,
So they turned themselves over to science.
As a result, they are released from their dependence on luck,
But not from their dependence on science

Hippocrates

The historical embedding of this thesis: lung transplantation in Groningen, the Netherlands

The research documented in this thesis was performed at the Cardiopulmonary Surgery Research Divison of the University Hospital of Groningen, in Groningen, The Netherlands.
Thirty years ago, in 1964 just after the first human lung transplantation, prof. Charles R.H. Wildevuur initiated a research program on lung transplantation that continues till today. The subjects of research in Groningen parallelled the international developments, and the Groningen group is now recognized as one of the leading groups in lung transplantation research.

The first problems encountered in lung transplantation concerned technical imperfections. In 1967 Charles Wildevuur published his thesis on the surgical and lung physiological properties of the transplanted lung, in which he showed that the early faillures of lung transplantation could be attributed to imperfect vascular and bronchial anastomoses, causing reduction in oxygen uptake and loss of graft function (1). In 1970 he published together with Benfield a review of the first 23 human lung transplants, which turned out to become one of the most cited articles on lung transplantation (2). In that article they stated that succesful human lung transplantation could only be achieved by better patient selection, by the development of a bilateral lung transplantation technique and better immunosuppression.

In 1980 de Langen published his thesis on the development of a bilateral lung transplantation technique (3). His thesis showed the feasability of this technique and showed that tissue matching of donor and recipient improves graft survival; indicating a solution of technical problems and a change to immunological problems after lung transplantation.

With the introduction of cyclosporine in the early 1980s' a new era of lung transplantation had started and although technical problems were still not fully solved, acute rejection of the lung transplant emerged as the major limiting factor for success. Elegant immunological research can only be achieved in inbred animals and a shift to the field of lung rejection research was initiated by Marck, who published in 1983 his thesis on a model of left lung transplantation in the rat (4). He showed the feasability of this technique and described some preliminary results on the mechanism of acute lung rejection.

The Groningen rat lung transplant model was made famous by the work of Jochum Prop, who published in 1984 his thesis on the mechanism of acute lung rejection (5). This work was the first systematic description of the factors related to acute lung rejection. His work is still among the most cited research on lung transplantion.

With this model and the use of cyclosporine long-term survival of rat lung transplants could be achieved. In 1991 Westra published her thesis on the factors inducing long-term survival of heart and lung transplants (6). She discovered the "combi-effect", in which co-transplanted lymphoid tissue in the lung (the BALT) together with cyclosporine induces some kind of tolerance of the lung transplant.

At present, most technical problems are solved, acute rejection can be treated relatively well, but the a new limiting factor in the further progress of lung transplantation is the development of immunologically mediated airway damage in long-term surving lung transplants. The present thesis deals with this problem.

As a result of the extensive experimental knowledge on lung transplantation, the University Hospital of Groningen was elected in 1990 to become the only transplant center for clinical lung transplantation in the Netherlands. At the moment of printing of this thesis 50 clinical lung transplantions have been performed in Groningen.

This thesis is a tribute to "the Groningen" Research and Clinical Lung Transplantation Program.

1. **Charles R.H. Wildevuur**. Longtransplantatie. Experimenteel-chirurgische en longfysiologische aspekten bij autotransplantatie. Thesis, Groningen 1967.
2. **Wildevuur Ch.R.H., Benfield JR**. A review of 23 human lung transplantations by 20 surgeons. Ann Thorac Surg 1970; 9: 489-515.
3. **Zacharias de Langen**. Simulataneous bilateral lung transplantation. Thesis, Groningen, 1980.
4. **Klaas Marck**. Long transplantatie bij de rat. Thesis, Groningen, 1983.
5. **Jochum Prop**. Lung allograft rejection in the rat. Thesis, Groningen, 1984.
6. **Albertine Westra**. The combi-effect of heart-lung transplantation in rats. Thesis, Groninen, 1990.

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Contents

1 Introduction

Though a simple curiosity today, the transplant of a gland may perhaps someday have a practical application
Alexis Carrel, Nobel Laureate 1901

The transplantation of lungs from one human being to another evolved in the second half of this century from a rare curiosity to an accepted treatment for end-stage pulmonary disease. Lung transplantations are now performed almost routinely world-wide and the results are steadily improving. However, the long-term results have not yet reached the same level of success as those of renal and cardiac transplantation (1, 2, 3). This lower success rate of lung transplantation is obvious from the survival curves in figure 1.

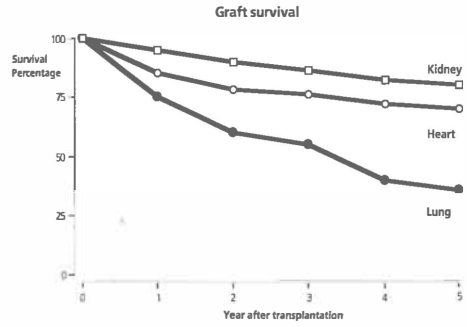


Figure 1.
Long-term succes after organ transplantation.
Although the results are improving, the long-term survival of lung transplants is still considerably lower than that of kidney and heart transplants.
Survival of lung transplants is the average of single, double, bilatelateral and heart-lung transplantation.

In this chapter we will describe the progress of lung transplantation in the past and the barriers that still prevent a high success rate today.

History

Lung transplantation has been the subject of an tremendous amount of investigative effort since the first experimental lung transplantation undertaken in 1907 by Alexis Carrel in his experiments on different types of vascular anastomoses (4). From the beginning it was clear that transplantation of the lung, as well as other solid organs, induces a variety of

technical, functional and immunological problems. The first studies in experimental lung transplantation were mainly concerned with solving technical problems and it was only in 1950 that Metras reported a technically successful lung transplantation in dogs (5). The cornerstone of his success was a change in the pulmonary venous anastomosis by suturing a left atrium cuff in stead of the isolated pulmonary veins. This technique decreased the incidence of pulmonary veinous thrombosis. Since then, this operative approach has been generally adopted and is also used in current clinical lung transplantation (6, 7, 8). The importance of perfect anastomosing techniques was further emphasized by Benfield (9) and Wildevuur (10, 11) who showed that after lung transplantation in dogs, elevated vascular resistance and poor lung function could be explained by structural defects at the site of the anastomoses of the blood vessels and bronchi.

With increasing numbers of experimental lung transplantations it became apparent that the large airway problems at the site of the bronchial anastomoses were to become the achilles' heel of lung transplantation (12, 13, 14, 15). All reports mentioned bronchial leakage and stenosis due to ischemic necrosis of the transplanted bronchus. It was evident that this poor healing of the bronchial anastomosis was the primary cause of failure of the lung transplant these experimental lung transplantations.

The first human lung transplantation was performed by Hardy and coworkers in 1963 (16). Although the patient died of renal insufficiency after 18 days, the lung transplant functioned well and without rejection. With this feat Hardy clearly demonstrated that human lung transplantation could be succesfully accomplished. The initial clinical results stimulated both experimental and clinical efforts throughout the world, and during the 20-year period that followed Hardy's first human lung transplantation, approximately 40 single lung transplantations were performed in patients with end-stage pulmonary disease. The results, however, were disappointing: the median survival was only 8.5 days and only

1.1 The progress of lung transplantation

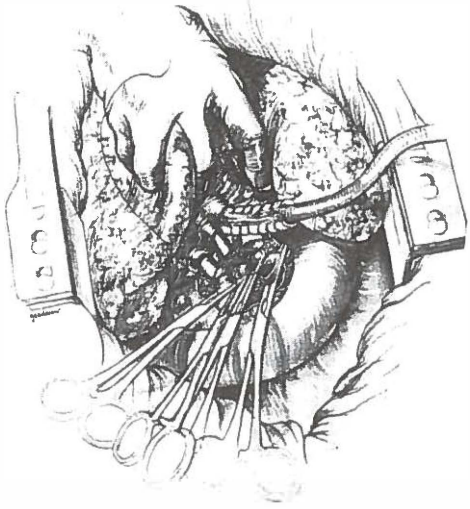


Fig 2.—Technique of lung transplantation in which anastomoses involving the two pulmonary veins, pulmonary artery, and main stem bronchus were performed. Sterile tube tied loosely into bronchus of donor lung permitted rhythmical ventilation of this organ with pure oxygen during the time it was without circulation.

Figure from the original paper by Hardy et al. describing the first human lung transplantation (ref 16) (With permission)

two patients survived for 6 and 10 months. In 1970 the results from the first 23 human, mainly unilateral, lung transplantations were analysed by Wildevuur and Benfield (17). They found that the poor results could be attributed to several factors: problems with the remaining diseased lung of the patient, technical problems resulting in bronchial dehiscense, poor lung preservation, infection and inadequate immunosuppression. Probably equally important in the early failures was the inappropriate selection of patients. Most of these transplanted patients were desperately ill with end-stage obstructive lung disease or malignancy. Wildevuur and Benfield therefore suggested that bilateral lung transplantation in combination with improved immunosuppression

could solve the above mentioned problems encountered in single lung transplantation. However, because neither a reliable double lung transplantation technique nor adequate immunosuppressive treatments were available at that time lung transplantation had been abandoned by all centers as a therapeutic option for patients with end-stage pulmonary disease by the mid 1970s'.

With the introduction of cyclosporine as a new and powerful immunosuppressive drug a new transplantion era started in the beginning of the 1980's. At the same time a reliable heart-lung transplantion technique had been developed in primates (18) and on March 9th 1981 the first human heart-lung transplantation in the cyclosporine era was performed by Reitz and Shumway from Stanford University (6). A new lung transplantation era had begun.

Evolution of techniques and indications

Lung transplantation can be performed in 4 different ways: transplantation of both lungs together with the heart, transplantation of a single lung and transplantation of both lungs. A fourth technique, the transplantation of a lung lobe from a living donor is currently evolving as a fourth option (19, 20) However, to date only 15 of these operations have been reported (19,20).

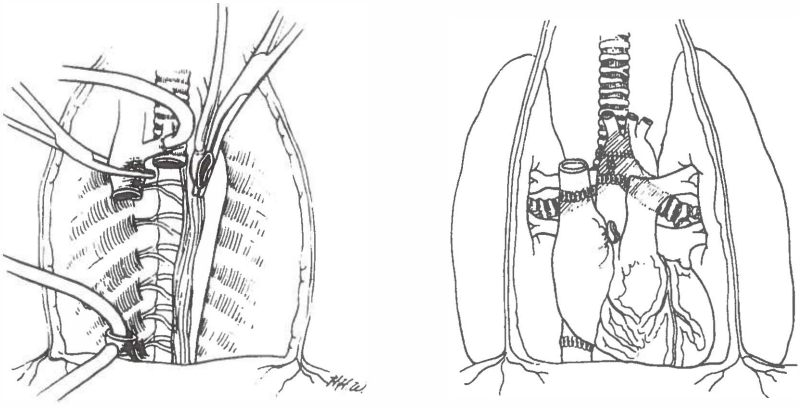
Since 1981 there has been a continuous change in indications for the different types of lung-transplantation. This evolution of indications will briefly be reviewed here.

Heart-lung transplantation

Between 1981 and april 1994, 1567 heart-lung transplantations have been reported to the Registry of the International Society for Heart and Lung Transplantation.

Clinical heart-lung transplantation evolved from the successes of heart transplantation at the end of the 1970s'. Heart transplantation was contraindicated for patients with pulmonary hypertension and these patients could only be treated by combined transplantation of a heart and 2 lungs. Consequently, between 1981 and 1985, most recipients of

heart-lung transplants had advanced pulmonary hypertension related to Eisenmengers' syndrome or primary pulmonary hypertension (6, 21). Gradually, an increasing number of patients with end-stage lung disease were succesfully treated with heart-lung transplantation. Especially patients with cystic fibrosis received heart-lung transplants (22, 23). The lungs of cystic fibrosis patients are in all cases chronically infected which needs removal and replacement of both lungs. Also in other end-stage lung diseases like emphysema transplantation of two lungs is indicated because otherwise the remaining lung would jeopardize the function of the transplanted lung (24, 25).



Procdure of heart-lung transplantation.
Figure on the left shows the thoracic cavity after removal of the recipient heart-lung block. Figure on the right shows the situation after completion of the tracheal, venae and aortic anastomoses.

Although heart-lung transplantation for end-stage pulmonary disease nowadays results in an excellent 5 year survival it has one major disadvantage: the transplantation of a heart in a recipient with a healthy heart of its own. Furthermore, a considerable number of long-term surviving heart transplants, with no exception for combined heart-lung transplants, develop accelerated coronary atherosclerosis caused by a chronic rejection process (26). This atheresclerosis ultimately leads to failure of the heart transplant (27). Another problem is the severe shortage of donors and combined heart-lung transplantation will waste a healthy heart (28). To overcome this problem some centers, especially Harefield in the UK, advocate the use of a so-called domino-transplantation of the removed heart from the recipient into a second recipient (29, 30). This procedure, however, has major logistic implications and in day-to-day practice the removed heart sometimes remains unused (30). It remains therefore controversial in the current era of organ shortage to sacrifice a perfectly healthy heart in recipients with diseases restricted to the lung (31).

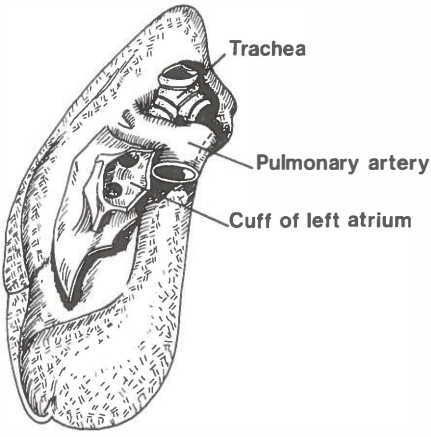
Single lung transplantation

Since most candidates for lung transplantation only suffer from diseases restricted to the lungs it was a logical approach to develop techniques for transplantation of the lung(s) without the heart (31).

Until 1983, no clinical survival of more than a year had been achieved by unilateral lung transplantation. Most of the early deaths had been related to inappropriate patient selection, infection, rejection and ischaemic disruption of the bronchial anastomosis. In November 1983 the first succesful single lung transplantation was performed by Cooper and coworkers from Toronto in a 58-year-old man with idiopathic pulmonary fibrosis (33). In the years that followed the Toronto lung transplantation program demonstrated that successful unilateral lung transplantation can be achieved after careful selection of patients (34, 35).

Till april 1994, 1943 single lung transplantations, performed

world-wide, have been reported to the Lung Transplant Registry.



Preparation of a single donor lung. A short segment of trachea and left bronchus remain in situ, as does a short segment of the main pulmonary artery.

As mentioned before, ischemic airway complications have been shown to be a frequent complication after lung transplantation and to be responsible for a considerable number of early deaths (15). The group of Cooper investigated in several studies the factors that impair bronchial healing: rejection, immunosuppression and bronchial ischemia. They showed that especially prednisolon had an adverse affect on bronchial healing (12). Further studies demonstrated the important role of ischemia in the donor bronchus due to interruption of its systemic bronchial arterial supply (13). The lung is the only solid organ transplant in which the systemic circulation (bronchial arteries) is not restored at the time of implantation, presupposing that the pulmonary circulation sufficiently perfuses the airways (36). Cooper showed that wrapping of the bronchus with a vascularized pedicle of the greater omentum resulted in early systemic bronchial circulation of the donor bronchus and significantly improved anastomotic healing

(37). Since then this technique was used by most transplant centers. However, more recent clinical experience (14, 15, 38, 39, 40) including a randomised trial from Harefield (31) have shown no difference in bronchial healing in patients with or without bronchial wraps. It is currently accepted that the occurence of airway complications after lung transplantation is influenced by the adequacy of preservation, the surgical technique and the early post-operative management. Results from experienced centers clearly indicate that the incidence of airway complications decreases with experience (14, 15, 38, 39, 40). So it seems that the bronchial anastomosis evolves, with increasing clinical experience, from the achilles' heel in lung transplantation into an infrequent post-transplant complication.

Single lung transplantation is performed in patients with fibrotic lung disease such as cryptogenic fibrosing alveolitis, sarcoidosis and eosinophilic granuloma (31,41). In those patients, reduced compliance and increased vascular resistance of the remaining fibrotic lung ensures a progressive shift of alveolar ventilation and lung perfusion from the native to the transplanted lung. In addition, the lungs of these patients are usually not infected.

In contrast, patients with emphysema were considered unsuitable recipients for single lung transplantation (17). This was based on the potential risk of overexpansion of the remaining emphysematous lung leading to compression of the transplanted lung. Furthermore, an early study on single lung tranplantation with this indication showed an increased perfusion with decreased ventilation of the transplanted lung, leading to an unacceptable pulmonary shunt (42). However, in a study from the Marseille group in France single lung transplantation appears to be surprisingly well tolerated in patients with end-stage lung emphysema (43). They showed with ventilation-perfusion scintigraphy that the lung allograft was adequately perfused and ventilated with an satisfactory ventilation-perfusion match. Also other major centers are currently strongly promoting single lung transplantation for

pulmonary emphysema (44, 45).

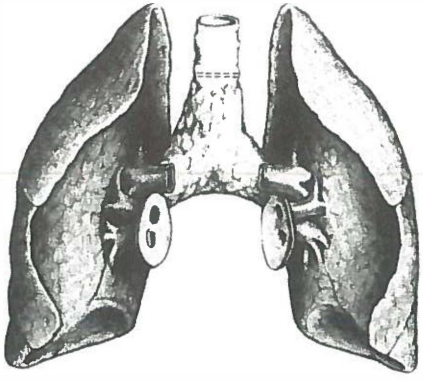
Single lung transplantation for primary pulmonary hypertension and Eisenmengers' syndrome is investigated by several groups (46, 47). However, single lung transplantation for this indication has certain disadvantages. It requires an excellent quality of the lung graft considering the fact that all cardiac output is directed to the transplanted lung. Second, rejection and infection can easily cause a ventilation-perfusion mismatch in these patients, thereby decreasing effective pulmonary function (47). The future will learn whether pulmonary hypertension can be adequately treated by single lung transplantation.

Single lung transplantation has several advantages over heart-lung and double lung transplantation. First of all, this procedure allows optimal organ utilization since the heart and the 2 lungs from one donor can be transplanted into 3 different recipients. Second, the risk of intra-operative and postoperative bleeding is lower since the surgical procedure is easier, the less affected side can be chosen in patients with pleural adhesions and cardiopulmonary bypass with its associated complications can often be avoided (49). Disadvantages of single lung transplantation are related to the remaining diseased lung, the smaller margin of functional reserve capacity during rejection and infection and possibly the smaller improvement in functional capacity (50).

Double and Bilateral lung transplantation

Patients with cystic fibrosis, emphysema and primary pulmonary hypertension may be suitable recipients for double lung transplantation, providing that right ventricular function is adequate or restorable. The Toronto group developped a succesful animal model for simulteneous, en bloc double lung transplantation (51, 52). In November 1986, the first human double lung transplantation in the cyclosporine era was performed in a patient with emphysema secondary to alpha-1-antitrypsisn deficiency (8). From 1986 untill april 1994, 943 double lung transplantations have been reported to the Registry.

During this procedure both lungs of the recipient are removed and replaced by the donor lungs. Initially, this en bloc double lung transplantation was performed with a tracheal anastomosis by most centers (8, 51). However, airway complications were extremely frequent after this type of operation. This made the Toronto group develop the technique of sequential bilateral lung transplantation (53).



Double lung block after removal from the donor. Lungs are then separated at the level of the bronchus and transplanted sequentially.

In this procedure the two lungs are transplanted sequentially and can be considered as two single lung transplantations in one patient. Sequential bilateral lung transplantation has two important advantages over en-bloc double lung transplantation. First, extracorporeal circulation and systemic heparinization can be avoided in most cases, because during transplantation of the first lung the body can adequately be ventilated by the remaining, although diseased lung (54). In the second stage of the operation the first transplanted lung takes over ventilation. The risk of bleeding is reduced in this way , which is in particular important in patients with cystic fibrosis because of the frequent dense pleura adhesions. A

second advantage is the reduction of ischemic airway complications. Two bronchial anastomoses appear to cause less airway problems than one tracheal anastomosis (15, 53, 54).

Compared to heart-lung transplantation, double and bilateral lung transplantation have the advantage of preserving the recipients' heart. This advantage in combination with the good results is now recognized by most of the lung transplant centers and during the last two years sequential bilateral lung transplantation has replaced heart-lung transplantation in most of the initial indications for heart-lung transplantation. Heart-lung transplantation is now largely restricted to patients with pulmonary vascular problems secondary to irreparable congenital heart diseases.

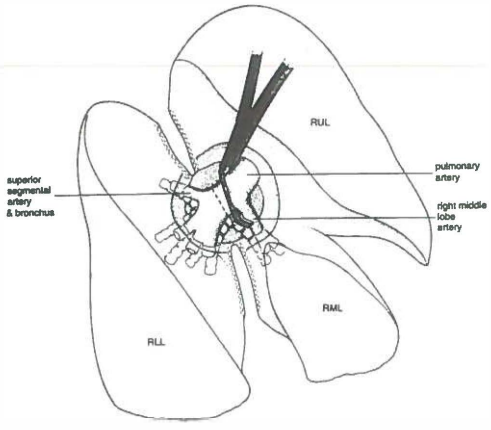
Lobar transplantion

Because of the severe shortage of donor lungs, transplantation of a lung lobe from a living donor could be a potential treatment for children with severe end-stage lung disease. This procedure would have two major advantages over the other types of lung transplantation. Since the donor is alive and in good health the operation can be timed and performed when the condition of the recipient has not fully deteriorated yet. Second, since the donor is preferably a close relative of the recipient the incidence of rejection may be lower than in unrelated transplantation. This advantage of living-related organ donation has been shown after kidney transplantation where a much better survival of the graft with reduced immunosuppression can be achieved (55).

Lobar transplantation from a living-related donor has now been performed in 7 children with systic fibrosis by Starnes from Stanford/UCLA Los Angelos (20, 56). Although lobar transplantation has some important advantages, it also has major disadvantages: it is a procedure with a potential mortality of 200%: both the donor and recipient are at risk and may die during or after the operation. Secondly, at this moment it is unclear whether transplantation of a grown-up lung lobe from an adult, with a fixed number of alveoli, will

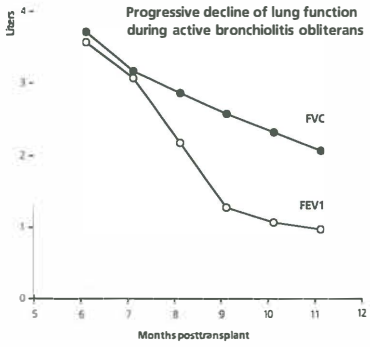
be able to grow in a child (57, 58, 59). Also, the ethical aspects of partial organ donation by living donors, whether relatives or unrelated (paid) donors, are still disputed. The International Society for Organ Transplantation has not taken a stand yet, but is very reluctant in approving this type of organ donation (60). The future will show if this type of operation will evolve from a somewhat controversial and heroic operation to a realistic option for end-stage pulmonary diseases.

Preparation of a living-related lobar transplantation.
The right lower lobe is divided from the right lung and prepared for implantation in the recipient



1.2 Bronchiolitis obliterans after lung transplantation

Lung transplantation is proving to be effective in the treatment of fatal and severe pulmonary disease. Nowadays, after lung transplantation many recipients are able to live a normal life and they can often resume their work (61, 62). At the beginning of the 1990s' most technical problems of lung transplantation are understood and can be managed adequately. Selection criteria of both donors and recipients become settled, operative techniques are refined and improved immunosuppressive agents permit control of acute rejection episodes. However, at the same time with increasing numbers of surviving lung transplants it has become clear that a great number of transplanted lungs fail within a few years after transplantation, despite continued immunosuppressive treatment (21, 23, 25, 26, 27, 63, 64). In fact, the results of long-term survival have not improved the last ten years. At present, in approximately 50% of the lung transplant recipients a clinical syndrome of progressive respiratory dysfunction develops (65). This progressive decline in lung function correlates with obstruction and destruction of small airways in the transplanted lung, known as bronchiolitis obliterans (BO).



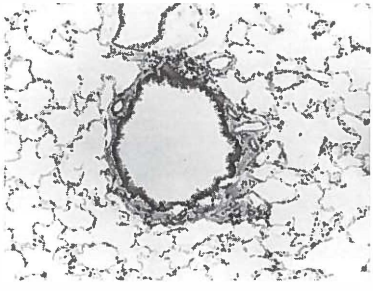
Progressive decline of the forced expiratory volume in one seconde (FEV1) and forced vital capacity (FVC) with time in a heart-lung transplant recipient with bronchiolitis obliterans. The patient did not respond to augmented immunosuppression (patient data from clinical data from chapter 4.2. Papworth Hospital Cambridge)

Initially, BO was only reported after heart-lung transplantation and was less frequently seen after single lung transplantation (64, 66, 67). Unfortunately, with increasing numbers of long-term surviving recipients of single and bilateral lung transplants it is now clear that BO affects the transplanted

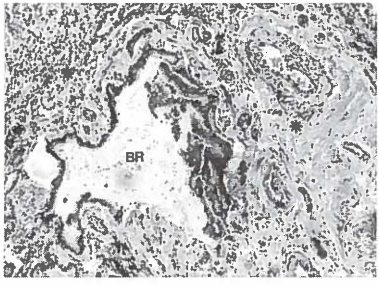
lung, irrespective of the type of lung transplantation (68, 69).

The first manifestations of BO may arise several months to 2 years after transplantation (64, 66, 67, 70, 71). Clinically BO is characterized by dyspneu, cough with or without sputum and chest tightness or wheezing (72). Physical examination may reveal persistent tachypneu and bilateral basal inspiratory crackles with occasional rhonchi. Pulmonary function tests reveal obstructive ventilatory impairment with progressive decrease in flow rates to very low values. Bronchodilatators have little or no effect on this airflow obstruction (66, 73). The increasing airflow obstructions leads to arterial hypoxemia (73). The chest X-rays may be normal or may show hyperinflated lung fields and basal periobronchial infiltration (74).

Although histopathological changes related to BO are usually found throughout the transplanted lung, the histological findings in the different areas of the lung may vary and may show different phases of the disease (75, 76, 77). In the earliest phases, usually observed in transbronchial biopsies, the changes consist of plugs of granulation tissue located in the lumen of terminal and respiratory bronchioles, associated with ulcerated bronchiolar epithelium and submucosal infiltration with chronic inflammatory cells (77, 78, 79, 80, 81).



Normal small airway (bronchiole)



Active bronchiolitis olbiterans,granulation and fibrotic tissue (*) are narrowing the bronchiole (BR)

Later, a scarring process develops and a wide range of luminal obstruction can be observed. A common feature is thickened

fibrotic submucosa, resulting in reduced luminal diameter and a rigid bronchiole. In the most severe cases, the bronchiolar lumen is entirely occluded by dense scar tissue.

Increased immunosuppression does arrest progression in some patients (82), but functional impairment related to BO is often inevitably progressive in most patients (83, 84, 85). The prognosis of of BO after lung transplantation remains consequently poor, and patients either die or are severely limited by dyspneu. At this stage retransplantation is the only chance of survival.

Unfortunately the etiology of BO in lung transplants is still unclear and as a consequence no therapy is available at this moment. However, parallells may be drawn with BO in non-transplant patients.

The etiology of bronchiolitis obliterans in non-transplant patients

Bronchiolitis obliterans is a disease affecting the small airways (bronchioles) of the lung. It is the histopathological end-stage of a process that starts with injury of the epithelium of the airways. Pathologically, BO shows a spectrum of airway changes ranging from from bronchiolar inflammation and mild scarring to complete obliteration of the bronchioles (86, 87, 88, 89). Two types of BO can be distinguished: bronchiolitis obliterans with organizing pneumonia (BOOP) and constrictive bronchiolitis obliterans (BO). BOOP is a combination of classic bronchiolitis obliterans (BO) with intraluminal polyps and organizing pneumonia (OP) affecting the alveolar septa and spaces. In contrast, constrictive bronchiolitis obliterans only affects the bronchioles and leaves the alveoli virtually unaffected. Both BOOP and constrictive BO are nonspecific tissue reactions that can be caused by a variety of triggers, without a clear relation between specific triggers and either BOOP or BO. Both entities can be seen within the lungs of one patient. An extensive review about this subject can be found in reference number 87. For the sake of simplicity we will not make a distinction between BOOP and BO and we will refer to BO in the rest of this paragraph.

Epithelial injury

The epithelial injury that leads to BO can be caused by a variety of factors like fume exposures, drugs, collagen diseases and allergic reactions. Most frequently, BO is observed following viral infections caused by respiratory syncytial virus, adenovirus, parainfluenza virus, mycoplasma, legionella, hemophilus, cytomegalovirus and chlamydia infections (86, 90, 91, 92). Most of the patients are young children and immunocompromised patients. A uniform finding in the lungs of these patients is the absence of neutralizing antibodies, especially IgA, suggesting that an inadequate pulmonary defense predisposes to BO (93, 94, 95).

Inflammation

Irrespective of the initial trigger, a non-specific inflammatory response is initiated after injury of the bronchiolar epithelium. Normally, this inflammatory response would eliminate the cause of the injury and simultaneously induce mechanisms that lead to healing of the damaged epithelium. The inflammatory response first shows a phase of acute bronchiolitis with invasion of macrophages and polymorphonuclear cells (PMNs) (87, 96). The macrophage is a versitale cell (or group of cells) with opposite effects: it is able to release oxidants, proteolytic enzymes and mediators (96,97), but also able to secrete antioxidants, antiproteases and inhibitors of cytokines . Macrophages are resident cells in the lung tissue (97). By contrast, the PMN is virtually absent from the alveoli in normal subjects (96,98). However, when recruited in inflammatory responses, the PMN can outnumber the macrophages and release substantial amounts of oxygen free radicals and proteolytic enzymes (96,97,98,99). Once they have arrived at the site of the epithelial damage macrophages and PMNs start to eliminate the damaged epithelial cells by release of oxygen free radicals and proteolytic enzymes. These release products cause necrosis of the epithelial cells which are then phagocytosed by the macrophages and eventually removed. At the same time the infiltrating macrophages induce tissue repair by production of cytokines (100). These cytokines attract fibroblasts and induce the production of collagen in the submucosa by these fibroblasts. In those cases where the inflammatory process remains activated, excessive

of fibrotic tissue will progressively grow into the lumen of the airway which becomes eventually obliterated, characteristic of BO. As result of the ongoing fibrotic changes the clinical course is usually marked by progressive airflow limitation eventually resulting in respiratory faillure and death (86, 87).

Lymphocytes are also seen in the inflammatory infiltrate, especially after viral infections (86,87). In this situation infection of the respiratory epithelium induces an anti-viral immune response by T-lymphocytes which then triggers a non-specific inflammatory reaction with the same sequence of events as described above.

The etiology of bronchiolitis obliterans in lung transplants

It is clear that the non-specific inflammatory response in the airways, characterized by the presence of macrophages and PMNs, is the key mechanism in the development of BO. In non-transplant patients, the trigger the inflammatory response can easily be identified in most cases. In lung transplant recipients, however, it is impossible in most cases to link the development of BO to a clearcut initial trigger.

Interfering factors

An important reason why the etiology of BO after lung transplantation is still unclear is related to the large number of interfering factors in the clinical situation. Even in one transplantation program, in one clinical center, immunosuppressive and antibiotic protocols cannot always be uniformely followed because of different reactions in the individual patient. One of the treacherous aspects of BO is that the clinical symptoms of pulmonary dysfunction appear in a late phase of the disease. As a result the initial cause of BO can usually not be identified in lung transplant patients. Various factors have been mentioned as most likely causes of BO in lung transplants, among which rejection and infection are most susptected (21-25, 31, 40, 45, 63, 64, 66-68, 72, 73, 75, 80-85, 101-105). Both rejection and infection can cause epithelial damage, which will initiate the inflammatory reaction that eventually leads to BO.

1.2 Bronchiolitis obliterans after lung transplantation

Rejection

Rejection, in particularly chronic rejection, is mostly considered to be the main cause of BO after lung transplantation to such an extent that it is used as an equivalent of BO in many publications. BO is believed to be the result of prolonged or repeated rejection episodes, especially affecting the bronchioles. This hypothesis seems te be supported by the observation of increased alloreactivity in cells lavaged from lung transplants with BO, although alloreactivity remains negative in a significant number of BO cases (106, 107). In experimental studies, BO-like airway damage was only found during severe end-stage acute rejection, a situation which is very uncommon after clinical lung transplantation (108, 109). Taken together, none of the clinical or experimental studies warrant to consider BO as the unequivocal result of a rejection process.

Infection

As described above, infections are an important of cause of epithelial damage and subsequent development of BO in nontransplant patients, especially in immunocompromised patients. Infections may play a similar role on the development of BO after lung transplantation. Infectious complications, especially affecting the transplanted lung, occur frequently after lung transplantation. Incidences have been reported to be as high as 90 % (110-115). This incidence of infections is much higher than after other organ transplantations despite comparable immunosuppressive protocols (111, 113). Early, within the first three months, after lung transplantation intrapulmonary bacterial infections cause pneumonia which result in post-transplantation mortality. After three months bacterial infections decline as a direct cause of death (114, 116, 117, 118), but the remaining viral infections that occur after three months cause high morbidity. Especially CMV infections, have been assumed to contribute to BO in many publications (66, 67, 83, 119, 120). CMV infections were found to preceed the development of BO in studies from Pittsburgh, showing upregulation of alloreactivity (120). However, studies from the Papworth group could not demonstrate such a clear influence of CMV on the development of BO (83). Besides CMV also respiratory tract

infections in lung transplant recipients have been suggested to be related with the development of BO (111, 115). This suggests that an aberrant immunological responses, i.e. reduced defense mechanisms, to these infections may cause BO by the same mechanisms as in non-transplant patients.

At this moment our understanding of the development of BO is limited and prevents the development of an adequate therapy. From the previous chapter it is clear that no distinct cause of bronchiolitis obliterans in lung transplants can be identified. Several causes are prominent candidates, but none is clearly the prime cause. The aim of this thesis is to investigate the causes and mechanisms that are involved in the etiology of BO in lung transplants. We hypothesize that the development of BO in lung transplants is caused by a combination of (chronic) rejection and infection.

To test this hypothesis we investigate in this thesis the contribution of chronic rejection and viral infection in the development of airway damage after lung transplantation in a rat model under standardised conditions. Furthermore, we investigate the possible mechanisms which lead to airway damage by analysing the immune responses associated with chronic rejection and viral infection in these lung transplants. The background of this study was introduced in chapter 1.2.

Chapter 2. Bronchiolitis obliterans in rat lung transplants

In chapter 2.1 we investigate whether isolated chronic rejection can cause bronchiolitis obliterans in long-term surviving rat lung allografts.

Since in our hypothesis bronchiolitis obliterans in lung transplants is caused by a combination of chronic rejection and infections we investigate in chapter 2.2 the influence of a respiratory viral infection on the development of BO is in the rat model of chronic lung allograft rejection as described in chapter 2.1.

To gain more insight into the mechanisms that lead to BO after lung transplantation we analyse in chapter 2.3 the immune and inflammatory responses associated with isolated chronic rejection, with isolated viral infection and with the combination of chronic rejection and viral infection in lung transplants.

Chapter 3. Immune responses in infected rat lung transplants

During the studies in chapter 2 in this thesis we find that respiratory viral infections severely aggravate the airway damage caused by chronic rejection in rat lung transplants. In this chapter we investigate whether an impaired defense against intrapulmonary antigens is responsible for this damaging effect of viral infections.

In chapter 3.1 we give a short overview of the defense system of the normal lung and its involvement in pulmonary infections in the normal lung, with emphasis on the function of the bronchus-associated lymphoid tissue (BALT) for an adequate pulmonary defense system.

In chapter 3.2 we investigate whether the systemic antibody response against an intrapulmonary antigen, sheep red blood cells, is impaired in rat lung transplants.

In chapter 3.3 we analyse the role of lymphatic drainage from the lung in a normal antibody response against intrapulmonary administered sheep red blood cells.

In chapter 3.4 we investigate the local antibody production in the BALT in the transplanted lung and the systemic antibody response in the blood after a respiratory viral infection in rats.

In chapter 3.5 we further analyse whether the integrity of the local defense system of the lung, the BALT, is preserved in rat lung transplants to allow an adequate immune response against respiratory infections.

Chapter 4. Immune responses in human lung transplants

We presume that BO in human lung transplants is caused by similar mechanisms as in rat lung transplants. In chapter 4.1 we suggest that the best material for analysis of immune responses in human lung transplants is obtained by transbronchial biopsy. In these biopsies immune responses initiated by rejection and infection can be investigated by immunohistochemical phenotyping of infiltrating cells.

As a first step in the analysis of immune responses in human lung transplants we investigate in chapter 4.2 whether cellular phenotyping can be used to detect immune responses in lung tissue obtained from human lung transplants with either acute or chronic rejection, without infection.

Chapter 5. Immunological causes of bronchiolitis obliterans after lung transplantation

In chapter 5.1 we give an overview of the possible causes and mechanisms involved in the development of BO in lung transplants, based on the findings in this thesis. We will integrate these findings into a model for the development of BO in lung transplants.

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2 Bronchiolitis obliterans in rat lung transplants

2.1 Late airway changes caused by chronic rejection in rat lung allografts

2.2 Respiratory infections aggravate airway damage caused by chronic rejection in rat lung allografts

2.2 Bronchiolitis obliterans in rat lung allografts is caused by aberrant inflammatory and immune responses against respiratory viral infections

2.1 Late airway changes caused by chronic rejection in rat lung allografts

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2.1 Airway changes caused by chronic rejection

Abstract

Airway disease after lung or heart-lung transplantation is one of the late major complications, affecting the prognosis of the transplants. Little is known about the causes of airway changes. We performed rat lung transplantation and investigated the late airway changes of the long-term surviving lung grafts: allografts; BN to Lewis, isografts; BN to BN rat. All recipients were treated with CsA. We found airway changes, i.e. mucosal ulceration, granulation, submucosal fibrosis, which was located in the large airways, in four of five allografted lungs. The lung isografts showed no pathological abnormalities.

Immunopathological studies disclosed the localized expression of MHC class II antigens on the bronchial epithelium of the large airways where recipient type dendritic cells accumulated in the submucosa and CD4 positive predominant lymphocytes infiltrated.

These findings support the idea that the late airway changes in lung transplants are caused by immunologically mediated chronic rejection.

Introduction

Airway disease, such as obliterative bronchiolitis, is one of the most common, late complications in the lung among recipients of heart-lung transplants (1). Obliterative bronchiolitis has been reported to occur also in patients with single lung transplants(2). Early pathology of the process shows injury of the bronchiolar epithelium in association with submucosal infiltrates of lymphocytes and accumulation of dendritic cells; later on, granulation tissue with acute and chronic inflammatory cells appears; eventually, dense scar tissue is formed that in severe cases occludes the entire lumen of the bronchiole.

The etiology of obliterative bronchiolitis in lung transplants is still uncertain. Various factors have been mentioned as conceivable causes, among which the surgical interruption of lymph vessels and bronchial arteries, infections (in particular viral infections (3)), and rejection. Chronic rejection has been alluded to be the most likely cause of obliterative bronchiolitis in several clinical reports(4). Information from clinical studies, however, is not very conclusive about the etiology of obliterative bronchiolitis because of the large number of interfering factors. Therefore, an animal model with more

standardized conditions would be helpful to investigate causes of obliterative bronchiolitis.

There are few animal studies, in which pathological changes similar to obliterative bronchiolitis have been observed. We found such changes in rat lung transplants during (severe) acute rejection(5). This was the first indication of an association between rejection and obliterative bronchiolitis. The changes in rats, however, differed significantly from those in patients in respect that they occurred during acute rejection, which also affected the alveolar structures.

Up to now, there is no description of an animal model of lung transplants with late airway changes associated with chronic rejection. The only hint for a chronic process in the airways of long-surviving rat allografts is the increased expression of class II MHC antigens locally on bronchial epithelium of the large airways at 100 days after transplantation (6). These antigens are indicative of an active immune process and have been hypothesized to serve both as stimulators and as targets of a rejection process resulting in localized epithelial damage.

In the present study, we analyzed whether the transplantation surgery or chronic rejection caused late airway changes in rat lung transplants in the absence of infections. The effect of surgery was investigated in syngeneically transplanted lungs. Chronic rejection was studied in allogeneic lungs; as symptoms of chronic rejection we assessed the expression of class II MHC antigens, submucosal distribution of dendritic cells, and infiltration of lymphocytes. Infection was excluded as a cause of airway changes by using rats free from viruses and pathogenic bacteria. All lung transplants were investigated by histology and immunohistology at six months after transplantation.

Materials and Methods

The left lung was transplanted orthotopically according to the technique of Prop and Marck et al(7). Syngeneic transplantation (n=5) was performed from BN to BN rats, and allogeneic transplantation (n=5) from BN to fully mismatched LEW rats. Both syngeneic and allogeneic recipients were treated with cyclosporin A (CsA), dissolved in olive oil and injected intramuscularly (25mg/kg) on the second and third days after transplantation (CsA was kindly provided by Sandoz, Basel, Switzerland). With CsA treatment, all allografted lungs were accepted. To exclude technical failures, the function of grafted lungs was monitored by chest roentgenography weekly at the first month and thereafter monthly till the day of sacrifice. The rats were killed under general anesthesia 6 months after transplantation.

Rats

Male specific pathogen-free inbred LEW: $RT1^{l}$ and BN: $RT1^{n}$, rats were obtained from the Zentral Institut für Versuchstiere, Hannover, Germany. They were separately housed from other rats to prevent infection with pulmonary viruses and pathogenic bacteria. Regularly samples were taken from the rat colony to confirm the absence of infections. Animal received humane care in compliance with the Dutch regulations and laws.

Histological studies

Heart and lungs were taken out en bloc from the thoracic cage. Cold (4° C) periodate-lysine-paraformaldehyde (PLP) fixative was infused intratracheally under +15 cm H_2O. The organs were immersed and kept for 3 hours. Both lungs were separated at the hilar portion and cut into two pieces through the main bronchus to get longitudinal bronchus sections. After evacuating the air from the lungs in a negative pressure box, one half lung was embedded for paraffin sections and the other half lung was snap frozen in liquid nitrogen and stored at -80° C. Paraffin sections were stained for light microscopy with hematoxylin and eosin (H&E), methyl green pyronine, and Masson's trichrome.

Serial cryostat sections were cut and air-dried for 30 min. The sections were rinsed in phosphate buffer saline (PBS) and then incubated at room temperature for 1 hour with the appropriate monoclonal antibodies. After washing 3 times in PBS, sections were incubated for 30 min with horseradish-peroxidase-conjugated rabbit anti-mouse immunoglobulin (DAKO, Denmark). Peroxidase was revealed by staining with 3,3'-diaminobenzidine tetrachloride. Sections were lightly counterstained with hematoxylin. To assess nonspecific staining, control sections were incubated with PBS instead of monoclonal antibodies.

Monoclonal antibodies

The monoclonal antibodies (MoAbs) used in the present study are listed in Table 1.

Antibody	Antigen and/or specificity
OX6	Anti-RT1.B., of all haplotypes
HIS19	Anti-RT1.B., $RT1^n$-negative (donor) and $RT1^1$-positive (recipient)
OX8	CD8 T cells, NK cells
ER2	Same as W3/25; CD4 T cells

The antibodies to MHC class II antigens were used to detect class II expression on the bronchial epithelium and observe the distribution of dendritic cells. We used the two different types of MoAbs to MHC class II antigens. HIS19 reacts with recipient type class II antigens but does not react with donor type class II antigens. That means that HIS19 positive cells in the allografted lungs are of recipient origin. OX6 reacts with both donor and recipient type class II antigens. To evaluate the subsets of infiltrating lymphocytes, we used ER2 for CD4 positive cells and OX8 for CD8 positive cells.

Results

Syngeneic transplants

Chest roentgenograms revealed no abnormalities in the transplanted left lung during the observation period. Syngeneic left lungs showed a macroscopic appearance similar to normal right lungs. There was no significant lymphocytic infiltration in the grafts. The bronchus was covered with intact normal epithelium and showed no fibrotic changes.

In immunohistology there was no class II expression on the bronchial epithelium through the airways of lung isografts. Dendritic cells were evenly distributed in the submucosa from the main bronchus till bronchioles, and no aggregates were seen.

Lymphocytes stained with CD4 and CD8 MoAbs were found in the BALT but there was no peribronchial or perivenous lymphocyte infiltation.

Allogeneic transplants

Serial chest roentgenograms revealed good aeration of the allografted left lung in all recipients during the observation period. The lung showed a nearly normal macroscopic appearance except mild pleural thickening and a few brownish spots on the pleural surface. Microscopically, lymphocytes, hemosiderin laden macrophages and occasional multinucleated giant cells were present subpleurally and around small veins. The alveolar structures were preserved without infiltration. Small airways showed intact bronchial epithelium but were accompanied mild lymphocytic infiltration in some part.

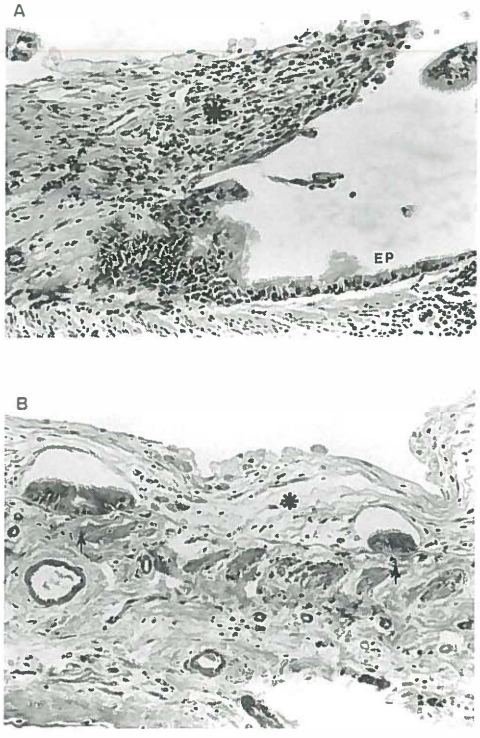
Remarkable changes were restricted to foci in the large airways, observed in four of five allografted lungs. In these foci epithelium was interrupted or ulcerated. There was focal infiltration of lymphocytes in the underlying submucosa. In some parts of the bronchus, excessive granulation tissue grew into the bronchial lumen and caused in narrowing the airway (Fig.1A). Islets of regenerative bronchial epithelium were found in the massive fibrotic tissue (Fig.1B).

Figure 1.

A. Photomicrograph showing granulation tissue protruding into the bronchial lumen.

B. Photomicrograph showing the islets of regenerative bronchial epithelium (arrows) in the extensive granulation tissue (asterisk).

EP: epithelium. H&E original magnification x200.



Immunohistologically, class II MHC antigens were detected on the bronchial epithelium of the large airways (Fig.2A). There was no expression of class II MHC antigens on the epithelium of small airways or bronchioles. Dendritic cells were increased in numbers and aggregated focally in the submucosa of large airways (Fig.2B). All dendritic cells were identified to be of recipient origin because they were stained by both OX6 and HIS19. Focal infiltrates of lymphocytes consisting predominantly of CD4 positive cells were found in the large airways.

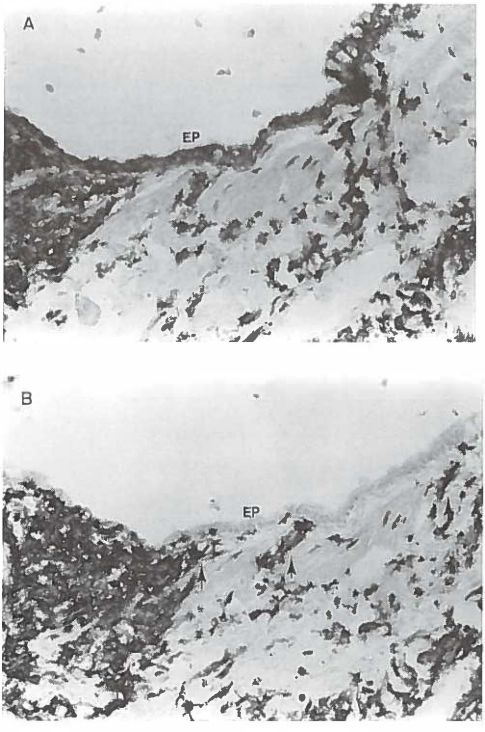


Figure 2. Large bronchus of allografted lung.

A. Immunoperoxidase staining of class II antigens with OX6; MHC class II antigens are expressed on the epithelium of the large airways.

B. Immunoperoxidase staining of MHC class II antigens with HIS19; HIS 19 positive cells with dendritic morphology (arrow) are increased in number and accumulated in the submucosa.

EP: epithelium. Original magnification x200.

Discussion

In the present study, we have demonstrated that late airway changes in long-term surviving rat lung allografts are caused by rejection.This is concluded from the finding that the airway changes, consisting of epithelial damage with granulation tissue and submucosal fibrosis, occurred only in allogeneic lung grafts. In addition, airway changes were confined to areas in the larger airways of these allografts in close association with the symptoms of chronic rejection.

Surgical interruption of lymph vessels and bronchial arteries does not cause airway changes in rat lung transplants: after syngeneic transplantation of the lung no abnormalities were found in the airways. Our experiments can not fully exclude the possibility that in larger animals and man the interruption of lymph vessels and bronchial arteries could result in more severe lymphatic congestion and ischemia of the bronchial tree and thus in the development of more significant airway changes.

Infection of lung transplantation is a factor that has been shown to increase the risk of development of late airway changes in clinical study (3). Any influence of infection was excluded in the present study by the use of infection-free rats, in order to reduce the number of factors contributing to the airway changes. Further research on the role of infections in the etiology of late airway changes is warranted, because they induce inflammatory responses in the lung transplant that closely resemble those of rejection, being the most important causal factor in our study.

The rejection process that we found to cause late airway changes is compatible with chronic rejection. It consisted of a triad of symptoms: MHC class II antigens expressed on the epithelium of the large airways; dendritic cells accumulated in the submucosa of the airways; and lymphocytes infiltrating in the submucosal tissue. Despite these symptoms, the allografted lungs were definitely accepted and continued to function. These symptoms have not been described before as a triad reflecting chronic rejection in association with late airway changes, they have merely been observed as isolated features.

Extensive expression of class II MHC antigens has previously been seen on the epithelium of rat lung allografts during acute, untreated rejection(6). All epithelium from the large bronchi till the small bronchioles became class II positive as soon as these structures were affected by the fulminant rejection process (5). With immunosuppression in the present and previous studies(6), class II MHC expression was reduced to the epithelium of large airways in the long-term surviving grafts. Now we have found that airways changes in these allografts were equally confined to the large airways. This is in parallel with the clinical observation that expression of class II MHC antigens is enhanced on the bronchial epithelium of patients suffering from obliterative bronchiolitis after heart-lung transplantation(8).

Accumulation of dendritic cells focally in the submucosa of allografts, as presently found 6 months after transplantation, has been investigated in a longitudinal study (9). In that study, it was shown that dendritic cells from the recipient replaced the donor cells and accumulated in the submucosa of the graft's large bronchi during the first postoperative month and then persisted for more than 3 months after transplantation. Because recipient dendritic cells can present alloantigens to lymphocytes (10), is was postulated that they pick up the MHC antigens expressed abundantly on the bronchial epithelium and present those to recipient lymphocytes, thus inducing a (chronic) rejection response. Similarly, Yousem et al.(4) have recently reported a significant increase in the number of - presumably recipient - dendritic cells in bronchial epithelium and submucosa of the donor lungs in patients with obliterative bronchiolitis.

Lymphocytic infiltration around the airways of long-term surviving rat lung allografts is less striking than around venules and so far little attention was pain to it. The association with class II positive epithelium has been mentioned earlier but not the epithelial damage as a result of chronic rejection. The fact that the infiltrating lymphocytes were predominantly CD4-positive cells is no a proof of a chronic rejection process, but also a recent report of chronic

rejection in kidney grafts, equally showed a predominant infiltration of CD4-positive cells (11).

Taking these observations together, we propose the following events lead to airway damage by chronic rejection. Recipient dendritic cells replace the donor cells in the submucosa. There, they pick up and process donor alloantigens, and present those to alloreactive lymphocytes. Upon this stimulation, cytokines are released that induce a vicious circle: expression of MHC alloantigens increase on epithelial cells; more dendritic cells are attracted, accumulate in the submucosa and process the abundantly available alloantigens; lymphocytes are recruited and activated by the alloantigens presented by dendritic cells. In this way, a local inflammatory response is initiated in which graft tissue, in particular bronchial epithelium, is damaged by antigen-specific and non-specific mechanisms (12). This sequence of events may be triggered by viral infections or periods of inadequate immunosuppression.

After the actual tissue damage, fibrosis and granulation tissue are replacing the inflammation in a healing process. In patients, this results in obliterative bronchiolitis as manifestation of end stage of chronic rejection in which the bronchial lumen is occluded by dense scar tissue. In rats, such scar tissue is absent but extensive fibrosis is seen in the affected airways. As the underlying mechanisms seem to be similar, the model of chronic rejection described in this paper may be used to investigate the contribution of the other factors, such as infections, in the development of obliterative bronchiolitis.

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2.2 Respiratory viral infections aggravate airway damage caused by chronic rejection in rat lung allografts

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2.2 Airway damage caused by chronic rejection and viral infection

Abstract

Airway damage resulting in bronchiolitis obliterans occurs frequently in patients after heart-lung and lung transplantation. Generally, chronic rejection is assumed to be the most important cause of bronchiolitis obliterans. However, viral infections might also be potential causes of airway damage after lung transplantation. In the present study we investigated whether viral infections could induce airway damage in rat lung transplants in the absence or in the presence of chronic rejection. We compared the histopathology of the airways in 3 groups of rats: group 1: non-transplanted LEW lungs, group 2: LEW-to-LEW syngeneic lung transplant and group 3: BN-to-LEW allogeneic lung transplants. Nontransplanted and transplanted rats were treated with cyclosporine A to induce permanent graft acceptance of the allografts. Six months after transplantation 4 non-infected rats of each group were killed for histological investigation (another 4 noninfected allografted rats were killed 56 days later) The remaining 16 rats in each group were infected with Sendai virus (parainfluenza type I) intratracheally. These rats were sacrificed for histological investigation 4, 7, 21 and 56 days after infection.

In the lungs of the noninfected rats of the nontransplanted and syngeneically transplanted groups airway changes were absent. After viral infection in these lungs, mild inflammation developed in the airways which was transient and completely resolved by day 56 after infection. In contrast, in the allogeneically transplanted lungs the viral infection caused severe and permanent damage of the airways. In the bronchioles and the large airways throughout the allogeneic lung transplants inflammation with epithelial necrosis and formation of granulation tissue was present. On day 56 after infection the bronchioles showed scarring in the submucosa and obliteration of the lumen: typical features of bronchiolitis obliterans.

This study shows that a respiratory viral infection aggravates the airway damage in rat lung allografts with chronic rejection. The findings suggest that viral infections and chronic rejection play a synergistic role in the development of bronchiolitis obliterans after human heart-lung and lung transplantation: the virus infection may stimulate chronic rejection and rejection may hamper the local defense against the virus.

Introduction

Bronchiolitis obliterans occurs frequently in patients after heart-lung and lung transplantation. In general, bronchiolitis obliterans is the end stage of a process that starts with epithelial injury of the airways followed by an inflammatory reaction that leads to formation of fibrosis in the submucosa and subsequently leads to obliteration of the airways. The epithelial injury can be induced by a variety of factors, like toxic chemicals, viral infections and autoimmune diseases (1).

In lung transplants the cause of the epithelial injury that leads to bronchiolitis obliterans is still unclear. The favored hypothesis is that chronic rejection is the prime cause of the epithelial injury (2,3). In several clinical studies potential causes of bronchiolitis obliterans after lung transplantation have been investigated, but the complexity of the clinical situation, with many interfering factors, does not permit a conclusive answer. More controlled conditions for investigation of the causes of bronchiolitis obliterans can be achieved experimentally in animals. In infection-free rats we showed that airway damage in lung allografts was caused by chronic rejection indeed (4). The airway damage in these rats, however, was mild and only present in the large airways. This was different from bronchiolitis obliterans after human lung transplantation where damage is severe and predominantly located in the smaller airways. Yet, these observations in animals were the first demonstration that chronic rejection alone can be the cause of airway damage in lung transplants.

Next to chronic rejection, viral infections have been put forward as a potential cause of airway damage already after the first observations of bronchiolitis obliterans in heart-lung transplant recipients (5,6). It is conceivable that viral infections in lung transplants directly damage the airway epithelium leading to bronchiolitis obliterans in a similar way as in non-transplanted lungs (1). Alternatively, viral infections might amplify the damaging effect of chronic rejection specifically in allografted lungs (6).

In the present study we investigated whether a respiratory viral infection could induce bronchiolitis obliterans-like

airway damage in long-term surviving rat lung transplants in the absence or presence of chronic rejection. To investigate the effect of a viral infection in the absence of chronic rejection we induced respiratory infections with Sendai virus in normal rats without lung transplants and in syngeneic lung transplants. To investigate the effect of viral infection in the presence of chronic rejection we induced the same infection in rats with allogeneic lung transplants showing mild airway damage caused by chronic rejection (4), Noninfected rats were investigated for comparison.

Materials and methods

Experimental design

Airway damage was investigated in long-term surviving rat lung transplants and in normal lungs with and without respiratory viral infection. LEW rats were divided in three groups (table 1).

Group	Noninfected		Infected			
	day 0	day 56	day 4	day 7	day 21	day 56
Normal LEW	4	-	4	4	4	4
LEW-to-LEW	4	-	4	4	4	4
BN-to-LEW	4	4	4	4	4	4

In group 1 the rats received no lung transplants (n=20), in group 2 the rats received syngeneic LEW lung transplants (n=20), and in group 3 the rats received allogeneic BN lung transplants (n=24) All rats were immunosuppressed with a CsA injection on days 2 and 3 after transplantation. The non-transplanted rats of group 1 received the same immunosuppressive treatment. Six months later 4 animals in each group were killed as non-infected controls (day 0). Additionally, 4 noninfected animals of the allogeneic group were killed 56 days later. The remaining 16 rats of each group

were infected intratracheally with Sendai virus (parainfluenza type I) At day 4, 7, 21 and 56 after infection 4 infected animals in each group were killed for histological examination of the airways.

Rats

Young adult, male, specific pathogen-free LEW ($RT1^l$) and BN ($RT1^n$) rats, weighing 250-350 grams were obtained from Zentral-Institut für Versuchstiere, Hannover, Germany. All animals received humane care in compliance with the Dutch regulations and law.

Lung transplantation

Left lung grafts were orthotopically transplanted in the thorax, according to the improved technique of Marck and Prop(7). Briefly, the donor lung was dissected and its vascular bed was flushed with cold saline. The recipient's left lung was removed and replaced with the donor lung; the pulmonary vein and artery were anastomosed first and than the bronchus.

To exclude technical failures, the function of transplanted lungs was monitored by chest roentgenography weekly during the first month and from then monthly untill the day of infection with Sendai virus. All chest roentgenograms showed normal appearence of the transplanted lung at the day of infection.

Immuno-suppression

All rats received cyclosporine-A (provided by Sandoz Pharmaceuticals Corporation, Basel, Switzerland), dissolved in olive oil, intramusculary in a dosage of 25 mg/kg body weight on day 2 and 3 after lung transplantation. This treatment is adequate to induce permanent graft acceptance of the allografts. Non-transplanted rats also received CsA for 2 days.

Viral infection

In this study Sendai virus (*Parainfluenza* type 1) was used to induce an intrapulmonary infection. The use of Sendai virus in experimental studies is well documented (8,9). Culture and preparation of Sendai virus were performed as previously described (9).

Six months after transplantation Sendai virus was injected intratracheally, at a dose of 10^3 placque forming units in 0.2 ml medium. In a pilot study with normal LEW rats this virus-load induced mild pulmonary changes (hyperplasia and lymphocytic infiltration of the bronchial epithelium and mild perivascular lymphocytic infiltration) which were transient.

Histology

For histological investigation of the lungs the rats were exsanguinated under ether anaesthesia. Heart and lungs were taken out en bloc from the thoracic cage. The lungs were intracheally infused with OCT (optimum cutting temperature) compound (Tissue-tek II: Lab-Tek Division. Miles Laboratories Inc. Naperville, IL) diluted 1:1 in PBS. Left and right lungs were separated at the hilar region and each lung cut into two pieces through the main bronchus to get longitudinal sections including the main bronchus. Lung halves were embedded for paraffin sections. Paraffin sections were stained for light microscopy with hematoxyllin and eosin (H&E) and Masson's Trichrome for fibrotic changes.

Results

Nontransplanted and syngeneically transplanted lungs

After infection of the nontransplanted and syngeneic transplants the airways showed mild and transient inflammation with little tissue damage. There were no differences between nontransplanted and syngeneically transplanted lungs, wich will be described together.

In the noninfected controls (*day 0*) the epithelium in the bronchioles and in the large airways was intact and showed no fibrotic changes (figure 1A). Also, no lymphocytic infiltrates were present.

On *day 4* after infection, the epithelium in the bronchioles and in the large airways was slightly hyperplastic. Both in the bronchioles and in the large airways the epithelium and the submucosa were focally infiltrated with low numbers of polymorphonuclear cells (PMNs) and lymphocytes, whereas peribronchial edema was absent.

On *day 7* after infection the airways reached its maximum of inflammatory changes. In the bronchioles and in the large airways the epithelium was more hyperplastic than on day 4, but it remained intact without necrosis (figure 1B). Both in the bronchioles and in the large airways the epithelium and the submucosa were focally infiltrated with moderate numbers of PMNs and lymphocytes, but peribronchial edema remained absent.

On *day 21* after infection, the airways had an almost normal appearance again: in the bronchioles the epithelium was normal whereas in the large airways the epithelium was still slightly hyperplastic. In parallel, both in the bronchioles and in the large airways the number of infiltrating PMNs and lymphocytes had almost disappeared from the submucosa.

On *day 56* after infection, the airways were entirely normal. No inflammatory airway changes and infiltrates were seen anymore (figure 1C).

In the right (nontransplanted) lungs in both groups the airways showed the same transient changes as in the left lungs and were also completely normal on day 56 after infection.

Allogeneically transplanted lungs

After infection in allogeneically transplanted lungs the airways showed severe inflammation and permanent damage.

In the noninfected lungs, the epithelium in the bronchioles had a normal appearance (figure 1D), whereas in the large airways the epithelium was focally ulcerated and denuded. In those foci granulation tissue protruded into the lumen of the large airways. Both around the bronchioles and the large airways lymhocytic infiltrates were present. In the bronchioles fibrotic tissue was absent in the submucosa, whereas in the large airways fibrotic tissue was present throughout the submucosa, but it left the muscular layer intact. The noninfected lungs at *day 56* showed similar histopathological changes as seen on *day 0*, with the only change that the layer of fibrotic tissue in the submucosa had thickened in the large airways.

On *day 4* after infection, the epithelium in most of the bronchioles and in the large airways was hyperplastic, but without necrosis. Both in the bronchioles and in the large airways PMNs and lymphocytes started to infiltrate in the submucosa in higher numbers than in the nontransplanted and syngeneically transplanted lungs on this time-point after infection.

On *day 7* after infection the inflammation became worse and gave more severe damage than in the nontransplanted and syngeneically transplanted lungs (figure 1E). The epithelium in most of the bronchioles was necrotic and denuded, with granulation tissue growing from the submucosa into the lumen. The lumina of the bronchioles were almost completely filled with an admixture of fibrin, mucus, necrotic epithelial cells and inflammatory cells, resulting in a significant obstruction of the lumen. In the large airways changes in the epithelium were less severe, but the epithelium was hyperplastic, metaplastic and focally necrotic. Granulation tissue was present in the submucosa , but without obstructing the lumen of the large airways. Simultaneously, both in the bronchioles and in the large airways the epithelium and the submusosa were infiltrated by high numbers of PMNs and lymphocytes. Only around the large airways peribronchial edema was present.

On *day 21* after infection, airway inflammation was still severe. In the bronchioles extensive inflammation with epithelial damage and granulation tissue was equally extensive as on day 7 after infection. The epithelium in the large airways was still diffusely hyperplastic and metaplastic with areas of epithelial necrosis together with formation of fibrosis in the submucosa. Both in the bronchioles and in the large airways the epithelium and submucosa were infiltrated by high numbers of inflammatory cells. The peribronchial edema around the large airways had disappeared.

On *day 56* after infection, the activity of the inflammation process was decreased, but had left the airways severely damaged. The epithelium in the bronchioles was dysplastic

Syngeneic lung transplants | **Allogeneic lung transplants**

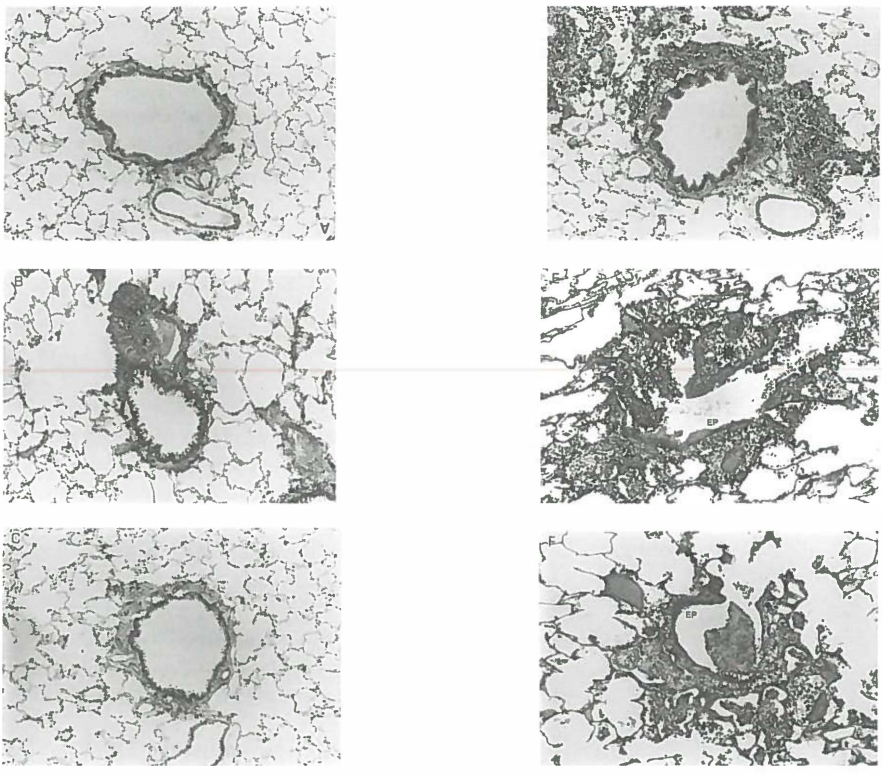

Figure 1. Sequential histology of bronchioles of syngeneic lung transplans (A, B, C) and allogeneic lung transplants (D, E, F) in noninfected rats (A and D), 7 days after infection with Sendai virus (B and E) and 56 days after infection (C and F) (hematoxylin and eosin, original magnification x 100; EP is epithelium)

Syngeneic lung transplants (A) Noninfected, normal bronchiole; (B) day 7 after infection: bronchiole with hyperplastic, but intact eptihelium, moderate numbers of PMNs and lymphocytes infiltrate in the submucosa; (C) day 56 after infection: normal bronchiole again.

Allogeneic lung transplants (D) Noninfected, bronchiole with intact epithelium and infiltration of lymphocytes in the submucosa; (E) day 7 after infection: bronchiole with destroyed architecture. The epithelium is interrupted and shows squamous metaplasia. Granulation tissue (asterisk) is protruding into the lumen. Large numbers of PMNs and lymphocytes infiltrate in the submucosa; (F) day 56 after infection: damaged bronchiole; a thin layer of metaplastic epithelium is only partially present. Scar tissue is present in the submucosa and a plug of myxoid connective tissue is olbiterating the airway. PMNs and lymphocytes are still present in the submucosa.

and extensive scarring was present in the submucosa. Intraluminal plugs of myxoid connective tissue obliterated the lumina of the bronchioles (figure 1F). In the large airways the damage was less extensive then in the bronchioles: the epithelium was still focally hyperplastic and throughout the submucosa fibrosis was present, but without obstructing the lumen of the large airways. Both in the bronchioles and in the large airways large peribronchial infiltrates of lymphocytes were present.

In the right (nontransplanted) lungs the airways showed the same transient changes as in the syngeneically transplanted and nontransplanted lungs and they were also completely normal on day 56 after infection.

Discussion

This study demonstrated that a respiratory viral infection induces severe damage in the airways of rat lung transplants, but only in combination with chronic rejection. The airway damage in the bronchioles of the allogeneic lung transplants showed the typical histopathological features of bronchiolitis obliterans: epithelial damage, formation of granulation and scar tissue in the submucosa and subsequent obliteration of the airway lumen (figure 1E and 1F). In contrast, in the absence of chronic rejection in the non-transplanted and syngeneically transplanted lungs, changes in the airways were only transient and the airways were completely normal 56 days after infection.

Bronchiolitis obliterans in lung transplants has emerged as an important late and often fatal complication after human heart-lung and lung transplantation. The etiology of bronchiolitis obliterans after human lung transplantation is still unknown. Several factors have been mentioned to induce the epithelial injury that leads to the eventual development of bronchiolitis obliterans. Among these factors the surgical procedure (interruption of bronchial blood flow, interruption of lymphatics and nerves) (10), chronic rejection (2) and viral infections (5) have been mentioned as most likely candidates.

Up to now the influence of these components on the development of airway damage could not be investigated separately, because no experimental model was available. Recently a model of chronic rejection in rat lung transplants was developed in our laboratory (4), which was used in this study to specifically investigate the influence of a respiratory viral infection superimposed on chronic rejection.

In the present study the surgical procedure did not influence the development of airway damage after viral infection. In syngeneically transplanted lungs, being exposed to the surgical procedure without influence of rejection, the same transient airway changes were seen after viral infection as in the non-transplanted lungs without the surgical trauma. In both groups the airways were completely normal 3 weeks after the viral infection. In our previous study we also oberved that the surgical trauma did not result in the airway changes as caused by chronic rejection (4). It shows that, at least in this rat model, the transplantation surgery does not play a noticeable role in the development of airway damage in lung transplants.

Chronic rejection is clearly a potential cause of airway damage, but its effect seems to to be less damaging in rats than in man. In the present study and in a previous study (4) in rat lung allografts, chronic rejection under infection-free conditions caused only mild airway damage in the large airways, whereas chronic rejection in human lung transplant recipients is supposed to cause severe bronchiolitis obliterans, throughout the small airways (2). This difference might be explained on one hand by a weaker immunological reaction against the allogeneic epithelium in rats than in man, but we are not aware of data to support this explanation. On the other hand the severe bronchiolitis obliterans in patients might indicate that in addition to the chronic rejection process other factors, such as infections, are involved in the development of airway damage.

The idea that viral infections contribute to the development of airway damage in lung transplants is

confirmed by the present study. We found the same histological airway changes after infection with Sendai virus in rat lung allografts as seen in human lung transplants with bronchiolitis obliterans. Whether other respiratory and pulmonary viral infections have a similar effect as a Sendai virus infection on the development of bronchiolitis obliterans remains te be shown. Possibly Sendai virus infects the bronchioles more directly than other viruses , because it is activated by the enzyme tryptase Clara that is produced by Clara cells in the bronchiolar epithelium (11).

The mechanism of airway damage by respiratory infection appears to be connected with rejection for obstructive features did not appear if viral infection was induced in the absence of chronic rejection, in both the non-transplanted and the syngeneically transplanted groups. A similar synergistic action of viral infections and chronic rejection has been seen in patients. In particular CMV infections have been reported to preceed the development of bronchiolitis obliterans in patients. The mechanism as proposed by Keenan and coworkers is that the viral infection amplifies the chronic rejection process by upregulation of lymphocyte alloreactivity (12). This is illustrated in that study by the observation that the donor-specific alloreactivity, detected by the primed lymphocyte test (PLT), increased following CMV infections. Similarly, Gryzan found an increased proliferative response of bronchoalveolar lavage cells against donor spleen cells after *Pneumocystis carinii* infection in heart-lung transplant recipients (13). This proposed mechanism that the infection-induced alloreactivity causes epithelial damage and so leads to bronchiolitis obliterans is compatible with the findings in the present study.

We propose a second mechanism through which respiratory viral infections may cause bronchiolitis obliterans. This mechanism is related to a reduced defense against the virus in transplanted lungs. The bronchus-associated lymphoid tissue (BALT) in the lung plays a central rol in the defense against pulmonary infections (14); e.g. the BALT is the place where most of the IgA is produced that protects the

airway epithelium in the lung from intrapulmonary pathogens. We have shown that the BALT is damaged and that it is replaced by fibrous tissue in long-term surviving rat lung allografts (15) and furthermore that the damage of the BALT hampers the anti-viral defense so that viruses are less adequately cleared form the lung (16). The persisting respiratory viral infection may have, first, a direct cytopathic effect on the epithelium of the airways and second, stimulate an ongoing inflammatory reaction. We hypothesize that these effects resulting from the reduced local defense contribute to the development of bronchiolitis obliterans in lung transplants.

Conclusion

Our findings support the hypothesis that also in patients with lung transplants viral infections play a role in the development of obliterative bronchiolitis and that these viral infections interfere with chronic rejection. In this perspective, a vigorous protocol of prevention, early detection and adequate treatment of viral infections as well as chronic rejection in patients after lung transplantation might help to reduce the incidence of bronchiolitis obliterans.

Acknowledgements
We like to thank Dr. J. van der Logt, ICLAS Reference Centre for Rodent Virusses, University of Nijmegen, The Netherlands for providing the Sendai virus used in this study and Dr. S. Welling, Department of Virology University Hospital Groningen and Drs. W. Hofstra, Central Animal Laboratory, University of Groningen, The Netherlands, for their virological advice in the design of the experiments.

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2.3 Bronchiolitis obliterans in rat lung allografts is caused by aberrant inflammatory and immune responses against respiratory viral infections

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Submitted

J Heart Lung Transplant 1994; 13; S46 (abstract)

2.3 Bronchiolitis obliterans caused by aberrant inflammatory and immune responses

Abstract

The etiology of bronchiolitis obliterans (BO) in lung transplants is largely unclear. Although chronic rejection and infection have been identified as most likely causes, little is know about the mechanisms by which they induce BO. Therefore, we analysed in this study the inflammatory and immune responses in the bronchioles induced by isolated chronic rejection, by isolated viral infection and by the combination of chronic rejection and viral infection in rat lung transplants. These responses were analysed histopathologically in 3 groups of rats before and after respiratory viral infection: group 1: BN-to-LEW (n=24) allografts, group 2: LEW-to-LEW (n=20) isografts, and group 3: normal (n=20) LEW rats. All rats were immunosuppressed with CsA (injected on days 2 and 3). Six months later 4 animals in each group were killed as non-infected controls and an additional 4 allografted rats were killed 56 days later. The remaining 16 rats in each group were infected intratracheally with Sendai virus and killed at days 4, 7, 21 and 56 after infection for immunohistological examination of the airways. Immunohistological indicators of the inflammatory response were inflammatory macrophages, oxygen free radical producing PMNs, NK cells and the expression of class II MHC antigens on the epithelium of the bronchioles. Indicators of the immune response were CD4 and CD8 T cells, with the IL2-receptor as activation marker and B cells.

In the bronchioles of allografts an aberrant and persisting inflammatory response, with infiltrating macrophages, PMNs and NK cells, was induced by the respiratory viral infection. Before infection, a minimal inflammatory response was caused by chronic rejection alone. Also, the immune response after infection was clearly abnormal in the allografts: in particular CD8 cells appeared slowly, but then persisted till the end of the observation period. In the syngeneically transplanted and non-transplanted lungs the inflammatory response was weaker while the immune response started immediately after infection; both responses were completely resolved 56 days after infection. The aberrant inflammatory response in the allogeneically transplanted lungs corresponded with early and severe epithelial damage in the bronchioles in these lungs.

This study shows that in rat lung allografts the combination of a respiratory viral infection and chronic rejection results in a severe aberrant inflammatory and abnormal immune responses that cause BO. It is conceivable that also in patients infection is the primary trigger of responses that eventually lead to BO.

Introduction

Bronchiolitis obliterans (BO) is the major problem affecting the long-term survival of lung transplant recipients. With increasing numbers of transplanted lungs (single, double and heart-lung transplants) the incidence of BO is increasing to 50% in lung transplant recipients surviving for more than 2 years (1,2).

The etiology of BO in lung transplant recipients has puzzled clinicians since the first observations in lung transplants (3), but parallells may be drawn with BO in non-transplant patients. There, the etiology of BO is related to a wide scale of causes of lung injury, such as infections, toxic fumes, allergic reactions and connective tissue diseases (4,5). All these causes lead to a response of non-specific bronchiolar inflammation, that eventually ends in BO. Pathologically, the inflammatory response is virtually independed of the primary cause, showing first an active phase of bronchiolitis consisting of infiltrates with macrophages, polymorhonuclear cells (PMNs) and lymphocytes and with epithelial damage (5,6). Later on the cellular infiltrates gradually disappear and tissue repair predominates. This is associated, however, with such an extensive formation of granulation tissue and fibrous tissue that the lumen of the bronchioles becomes partially or completely occluded. Although clinical symptoms of pulmonary dysfuntion appear in a late phase of BO, in non-transplant patients the primary cause of BO can usually still be identified. In lung transplant recipients, however, the cause of BO remains unclear in most cases (7). Based on earlier publications of BO occuring after bone marrow transplantation, rejection and infection were generally regarded to be the causes of BO after lung transplantation (8,9,10).

Rejection, in particular chronic rejection, is generally considered to be the main cause of BO after lung transplantation and to such an extent that it is used as an equivalent of BO in many publications (7,9). In this view BO is believed to be the late result of prolonged or repeated rejection episodes affecting the bronchioles specifically (7).

This view seems to be supported by the observation of increased alloreactivity in cells lavaged from lung transplants with BO, although alloreactivity remains absent in a significant number of BO cases (11). Also, a few animal studies indicated a correlation between rejection and BO. During severe (end-stage) acute rejection of rat lung transplants we found changes similar to those of BO (13,14). In contrast to BO in patients, however, these changes in rat lungs were not restricted to the airways, but they also involved the vessels and the alveolar compartment of the lung. More restricted airway changes were found during chronic rejection of lung transplants in CsA treated rats, with focal granulation tissue protruding into the lumen, but these chronic changes were only mild and left the bronchioles unaffected (15). Taken together, none of the clinical or experimental studies warrant to consider BO as the unequivocal result of a rejection process.

Also infections, especially CMV infections, have been assumed in many publications to contribute to BO (7,9,10). CMV infections were found to preceed the development of BO in studies from Pittsburgh, showing upregulation of alloreactivity (10). However, other clinical studies could not demonstrate such a clear influence of CMV on the development of BO (7,9). In an animal study we convincingly showed that in rat lung transplants viral infections indeed played a role in the development of BO (16). In that study, a respiratory viral infection aggravated the airway damage caused by chronic rejection: the airway damage extended from the large airways into the bronchioles. Two mechanisms were proposed for this damage, based on the fact that the BO-like changes only occured in allogeneic and not in syngeneic rat lung transplants. First, the viral infection might enhance graft-specific alloreactivity, as was seen previously in clinical studies after CMV and pneumocystis infection (10,17). Second, the viral infection might generate a persisting inflammatory response because the virus can not be cleared adequately from the allograft (18). This second mechanism would imply that an aberrant response is important in the generation of post-transplant BO.

In this study we analysed how the response against a mild respiratory viral infection progresses into a destructive process causing BO in all the infected lung allografts in rats (16). We haven chosen a histopathological assessment of the cellular responses in the airways, recognising the inflammatory arm and the immune arm of the response. We realise that the inflammatory and immune arm interact in a complex way during antiviral and rejection responses, and that besides cells also cytokines play a role. For simplicity, however, we defined the inflammatory response as infiltration by macrophages, PMNs with increased superoxide anion (O_2^-) production, and NK cells, together with expression of class II MHC antigens on the bronciolar epithelium. These inflammatory cells are part of the innate immune system, which is fully functional and can react immediately when a pathogen enters the lung. The immune response was defined as infiltration by CD4-positive and CD8-positive T cells, with the IL2-receptor as activation marker, and B cells. These immune cells belong to the adaptive immune system, and need to be activated before they can react against specific antigens. These definitions were used to classify the responses in the bronchioles of the transplanted rat lungs.

First, the inflammatory and immune responses in lungs with and without chronic rejection were assessed in long-term surviving noninfected allogeneic and syngeneic rat lung transplants, and in nontransplanted lungs, respectively. Next, to assess the infection-induced inflammatory and immune responses, rats with allogeneic and syngeneic lung transplants and nontransplanted rats were infected with Sendai virus. The inflammatory and immune responses in the bronchioles were assessed at different time-points after infection by analysing phenotypes of infiltrating cells together with parameters of activation. We found that the intensity and time-course of both inflammatory and immune responses in allogeneic lung transplants were highly abnormal when compared with syngeneic lung transplants and nontransplanted lungs.

Materials and methods

Experimental design

The inflammatory and immune responses were investigated in the bronchioles of long-term surviving lung transplants and nontransplanted lungs after intrapulmonary infection with Sendai virus (parainfluenza type I). LEW rats were divided in three groups. In group 1 the rats received allogeneic BN lung transplants (n=24), in group 2 the rats received syngeneic LEW lung transplants (n=20), and in group 3 the rats received no lung transplants (n=20). All rats were immunosuppressed with a CsA injection on days 2 and 3 after transplantation. The non-transplanted rats of group 3 received the same immunosuppressive treatment. Six months later 4 animals in each group were killed as noninfected controls. The remaining 16 rats of each group were infected intratracheally with Sendai virus. At days 4, 7, 21 and 56 after infection 4 infected animals in each group were killed for immunohistological examination of the airways. Additionally, 4 noninfected animals of the allogeneic group were killed 56 days after infection of the other rats as noninfected controls. The phenotypes of infiltrating inflammatory and immune cells were determined by immunohistology. Parameters of the inflammatory response were an increase of (inflammatory) macrophages, PMNs with increased superoxide anion (O_2^-) production, and NK cells and the expression of class II MHC antigens on the epithelium of the bronchioles. Parameters of the immune response were an increase of CD4-positive and CD8-positive T cells, with the IL2-receptor as activation marker, and an increase of B cells.

Rats

Young adult, male, specific pathogen-free LEW ($RT1^l$) and BN ($RT1^n$) rats, weighing 250-350 grams were obtained from Zentral-Institut für Versuchstiere, Hannover, Germany. All animals received humane care in compliance with the Dutch regulations and law.

Lung transplantation

Left lung grafts were orthotopically transplanted in the thorax, according to the improved technique of Marck and Prop (19). Briefly, the donor lung was dissected and its vascular bed flushed with cold saline. The recipient's left lung was removed and replaced with the donor lung; the pulmonary vein and artery were anastomosed first and than the bronchus.

To exclude technical failures, the function of the transplanted lungs was monitored by chest roentgenography weekly during the first month and then monthly untill the day of infection with Sendai virus. All chest roentgenograms showed normal appearance of the transplanted lung at the day of infection.

All rats received cyclosporine A (provided by Sandoz Pharmaceuticals Corporation, Basel, Switzerland), dissolved in olive oil, intramuscularly in a dosage of 25 mg/kg body weight on day 2 and 3 after lung transplantation. This treatment is sufficient to induce permanent graft acceptance of the allogeneically transplanted lungs. Nontransplanted rats also received CsA for 2 days.

Virus

In this study Sendai virus (*Parainfluenza* type I) was used to induce a respiratory infection. Culture and preparation of Sendai virus were performed by the ICLAS reference center for rodent viruses (University of Nijmegen, Nijmegen, The Netherlands) as previously described (16, 20). The Sendai virus was injected intratracheally, at a dose of 10^3 PFU in 0.2 ml medium. In a pilot study this virus-load induced mild pulmonary changes in normal LEW rats with hyperplasia and lymphocytic infiltration of the bronchial epithelium and mild perivascular lymphocytic infiltration, that resolved in 4 weeks.

Lung tissue

For histological and immunohistological investigation of the lungs the rats were exsanguinated under ether anaesthesia. Heart and lungs were taken out from the thoracic cavity. The lungs were intracheally infused with OCT (optimum cutting temperature) compound (Tissue-tek II: Lab-Tek Division. Miles Laboratories Inc. Naperville, IL) diluted 1:1 in PBS.

Left and right lungs were separated at the hilar region and each lung was cut into two halves through the main bronchus to get longitudinal sections including the main bronchus. One lung halve was embedded for paraffin sections, the other lung halve was snap frozen in liquid nitrogen and stored at -80°C for immunohistological evaluation. Paraffin sections were cut at 6 µm and stained for light microscopy with hematoxillin and eosin (H&E).

Immuno-histology

Monoclonal antibodies were used in this study to determine the phenotypes of infiltrating cells in non-infected and infected lungs. To determine the inflammatory response monoclonal antibodies were used that recognised macrophages (ED1), inflammatory macrophages (ED2), PMNs (HIS 48), NK cells (α-ASIALO) and MHC class II antigens on bronchiolar epithelium (OX6). To determine the immune response monoclonal antibodies against CD4-positive (ER2) and CD8-positive (OX8) T cells, IL2-receptor (OX39) and B cells (HIS14) were used.

For immunoperoxidase staining, serial cryostat sections (thickness 6 µm) were cut and air-dried for 30 min. The sections were rinsed in PBS and then incubated at room temperature for 1 hour with the appropriate monoclonal antibodies. After washing 3 times in PBS, sections were incubated for 30 minutes with horseradish-peroxidase-conjugated rabbit-antimouse immunoglobulin (DAKO, Denmark). Peroxidase was revealed by staining with 3,3'- diaminobenzidine-tetrachloride (Sigma, Germany) dissolved in PBS at a concentration of 1 mg/ml PBS containing 0.02% H_2O_2. Sections were lightly counterstrained with hematoxylin. To assess nonspecific staining, control sections were incubated with PBS instead of monoclonal antibodies.

The presence of superoxide anion producting PMNs was demonstrated in lung cryostat sections by the method of Briggs (21) applied at the light microscopical level. This method is based upon the oxidation of Mn++ to Mn+++ by O_2^- and the subsequent oxidation of Dulbecco's A+B medium (DAB) (Oxoid, Basingstoke, England) by Mn+++. Inhibition

of staining by addition of 300 U/ml superoxide dismutase (SOD) (Serva, New York) to the incubation media confirms the demonstration of O_2^- production by this method.

Cell scores

Positive cells around the bronchioles were scored in a semiquantitative way, recognising 4 infiltration scores in which - = no cells, + = low, ++ = intermediate and +++ = high number of positive cells per field of view at a magnification of x400. At least four fields containing bronchioles in each lung section were examined. The ordinal data of the infiltration score are presented graphically as means ± SE for simplicity of interpretation. Comparisons between groups were made with the Mann-Whitney rank sum test for ordinal data. All calculations were performed with the statistical software package Statview II for the Apple Macintosh computer. A p value of less than 0.05 was considered to indicate statistical significance. For readability of the text, stastical significant differences are only mentioned in the legends of the figures.

Results

Because the results of the syngeneically transplanted and nontransplanted groups were similar for histological, inflammatory and immune parameters they will be presented together.

Histology

Only the combination of chronic rejection and viral infection caused severe BO-like airway damage. In the noninfected allogeneneically transplanted lungs with chronic rejection, the epithelium in the bronchioles had a normal appearance six months after transplantation, although in the submucosa lymphocytic infiltrates were present. After infection severe airway damage developed. On day 4 after infection, the epithelium in most of the bronchioles was hyperplastic, but without necrosis. On day 7 after infection the epithelial damage in the bronchioles reached its maximum, with epithelial necrosis and formation of granulation tissue protruding into the lumen (fig 1A). The submucosa was

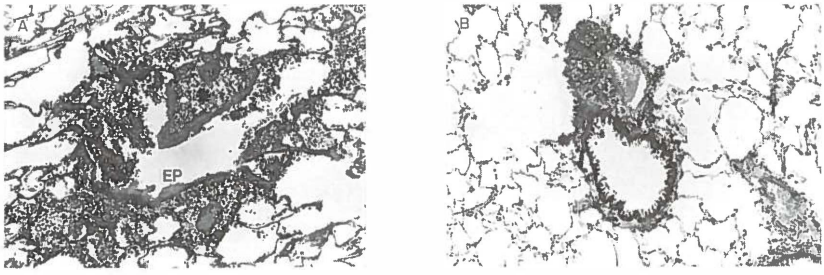


Figure 1. Airway damage on day 7 after viral infection in allogeneically transpalnted lungs (A) and syngeneically transplanted lungs (B). (H&E, original magnification x100; EP is epithelium.

Allografts A. On day 7 after infection the epithelial damage reached its maximum, with epithelial necrosis and formation of granulation tissue (asterisk) protruding into the lumen of the bronchioles. The submucosa was infiltrated with large numbers of macrophages and PMNs.

Isografts B. The airway damage in these lungs was only mild, with maximum changes on day 7 after infection: the epithelium was hyperplastic, but intact without necrosis. Moderate numbers of macrophages, PMNs and lymphocytes were present in the submucosa.

infiltrated with large numbers of macrophages and PMNs. On day 21 extensive epithelial damage and granulation tissue was still present, with high numbers of macrophages, PMNs and lymphocytes infiltrating into the submucosa, the pathological picture of chronic inflammation. On day 56 the activity of the chronic inflammation process was diminished, but had left the bronchioles severely damaged: the bronchioles showed scarring in the submucosa and obliteration of the lumen. Peribronchiolar infiltration of macrophages and lymphocytes was reduced, but it remained more prominent than prior to infection.

In contrast, the airway changes in the syngeneically and nontransplanted lungs were mild and only transiently present. In the lungs of the noninfected rats the epithelium of the bronchioles was always normal, without lymphocytic infiltration. After viral infection in these lungs, mild damage developed in the bronchioles with maximum changes on day 7 after infection: the epithelium was hyperplastic, but remained intact without necrosis (fig 1B). Moderate numbers

of macrophages, PMNs and lymphocytes infiltrated into the submucosa. On day 21 after infection the epithelium had an almost normal appearance again. In parallel, infiltrating macrophages, PMNs and lymphocytes had almost disappeared from the submucosa. On day 56 the bronchioles were entirely normal without epithelial changes or cellular infiltrates.

Inflammatory response

Macrophages
Figure 2 and 3.

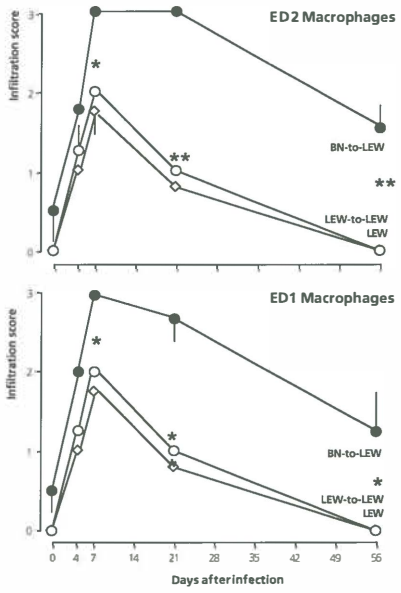
Before infection, ED1 positive cells were only present around the airways of the allogeneically transplanted lungs whereas they were absent in the syngeneically transplanted and nontransplanted lungs. After infection their numbers increased in all 3 groups, reaching a peak on day 7 and declining thereafter. At the end of the observation period ED1 positive macrophages were present in higher numbers in the allogeneic lung transplants than prior to infection, but they were absent again in the syngeneic lung transplants and normal lungs.

Figure 2. Infiltration of macrophages

ED1 macrophages. Before infection ED1 macrophages were only present in the airways of the allogeneically transplanted groups. After infection their numbers increased in all 3 groups, reaching a peak on day 7 and declining thereafter. From day 7 onwards their numbers were highest in the allografts.

ED2 macrophages. Before infection few ED2 macrophages were only present in the allografts. Immediatly after infection they started to infiltrate around the bronchioles in all 3 groups, reaching a peak on day 7 and declining thereafter. The peak and following decline were more pronounced in the allografts.

(* = p<0.05, ** = p<0.01)



Few ED2 positive inflammatory macrophages were present around the airways of the non-infected allogeneically transplanted lungs, whereas they were completely absent in the non-infected syngeneically transplanted and non-transplanted lungs. Immediately after infection, ED2 positive macrophages started to infiltrate the bronchioles in the lungs of all 3 groups. Their numbers reached a peak at day 7, but the peak was higher and the following decline was slower in the allogeneically transplanted lungs than in the syngeneically and non-transplanted lungs.

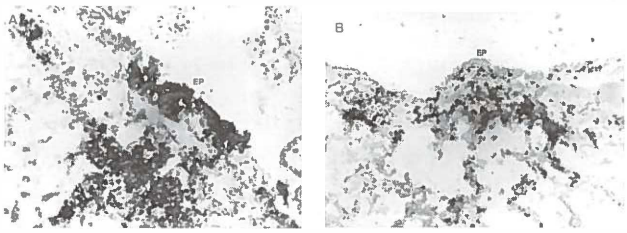


Figure 3.
ED2 macrophages
Day 7 after infection

A. allografts
B. isografts

Magnification x200.
EP is epithelium

PMNs
Figure 4 and 5.

The PMNs showed a similar pattern as the ED2 positive macrophages. They were absent in the non-infected lungs. After infection, the numbers in the bronchioles continued to rise untill day 7 in the allogeneically transplanted lungs and they did not fully disappear from the allografts in the following period. In the syngeneically transplanted and non-transplanted lungs, PMNs also had their peak already on day 7, but they were absent again from day 21 onward.

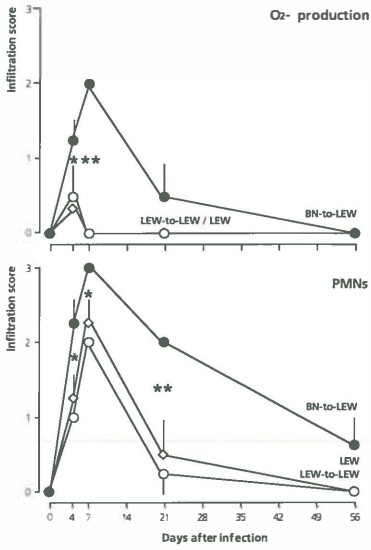
O_2^- production, as marker of PMN activation, was not seen in the noninfected airways of any of the 3 groups. After viral infection, high numbers of O_2^- producing cells appeared around the bronchioles in the allogeneically transplanted lungs. In the syngeneically transplanted and nontransplanted lungs a low peak was seen only on day 4.

Figure 4. Infiltration of O^{2-} producing PMNs.

PMNs. Infiltration of PMNs showed a similar pattern as the ED2 macrophages. After infection they started to infiltrate around the airways in all lungs. Their numbers continued to rise in the allografts, reaching a peak on day 7, declining afterwards, but remaining present. Peak numbers in the isografts and nontransplanted lungs were lower and the following decline steeper. Numbers of PMNs were higher in the allografts on day 4, 7 and 21.

O_2^- production. O_2^- production as a marker of PMN activation was absent before infection in all lungs. After infection O_2^- producing cells appeared in all lungs, but their numbers were higher in the allografts.

(* = p<0.05, * = P<0.01)



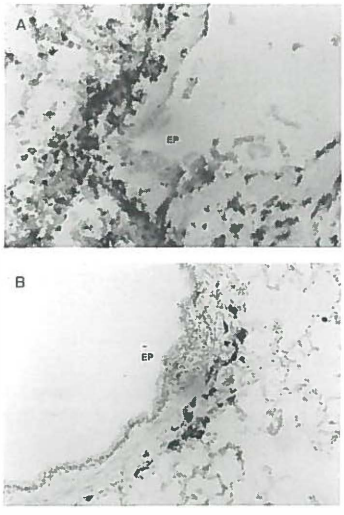


Figure 5.
O_2^- producing PMNs
Day 4 after infection

A. Allografts
B. Isografts

Magnification x200
EP is epithelium

NK cells Figure 6.

In the allogeneically transplanted lungs NK cells rose slowly after infection to low numbers on day 21 and remained constant thereafter untill the end of the observation period. In contrast, the number of NK-cells in the syngeneically transplanted and non-transplanted lungs increased immediately after infection , reaching a peak on day 7 after infection, and then declined to zero at the end of the observation period.

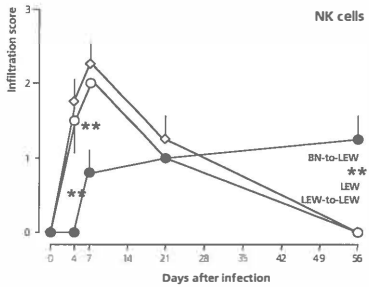


Figure 6. Infiltration of NK cells.

In the allografts NK cells rose slowly after infection to low numbers on day 21 and remaining constant thereafter. In the isografts and nontransplanted lungs NK infiltration showed an inverse pattern, with an early peak and decline thereafter.

(** = p<0.01)

MHC class II Figure 7.

In non-infected lungs MHC class II antigens were not expressed on the epithelium of the bronchioles in any of the 3 groups. After infection the small airway epithelium started to express MHC class II antigens intensively in the allogeneically transplanted lungs that continued up till the end of the follow-up period. In the syngeneically transplanted and nontransplanted lungs MHC class II antigens were expressed weakly at 4 days and remained expressed for a few weeks, but had disappeared by day 56.

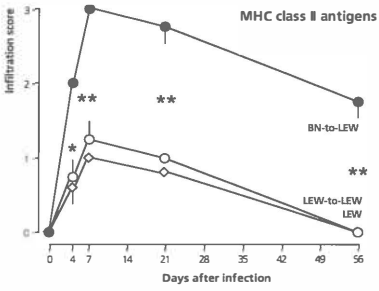


Figure 7. MHC class II expression on bronchial epithelium.

Before infection class II antigens were not present in any of the 3 groups. After infection the small airway epithelium started to express class II intensively in the allografts, that continued up till day 56. In the other lungs class II expression was less intense and had disapeared at day 56.

(* = p<0.05, ** = p<0.01)

Immune response

T cells

Figure 8 and 9. Already in the noninfected allografts, CD4-positive and some CD8-positive cells were present around the bronchioles, whereas they were absent in the noninfected syngeneically transplanted and non-transplanted lungs. After infection the number of CD4- positive cells increased sharply within 4 days in the lungs of all 3 groups. Their numbers remained high in the allogeneically transplanted lungs untill the end of the observation period. In contrast, in the syngeneically transplanted and nontransplanted lungs CD4-positive cell numbers dropped gradually after day 7 to zero by day 56. The pace of infiltration of CD8-positive cells was remarkably slow in the allogeneic transplants: CD8-positive cells increased steadily to reach its maximum only by day 21 and then they remained present in these lungs untill the end of the observation period, almost similar as the influx of NK cells. In the syngeneically transplanted and non-transplanted lungs the CD8-positive cells increased sharply after infection, reaching a high peak on day 7 after infection, dropping thereafter to disappear by the end of the observation period.

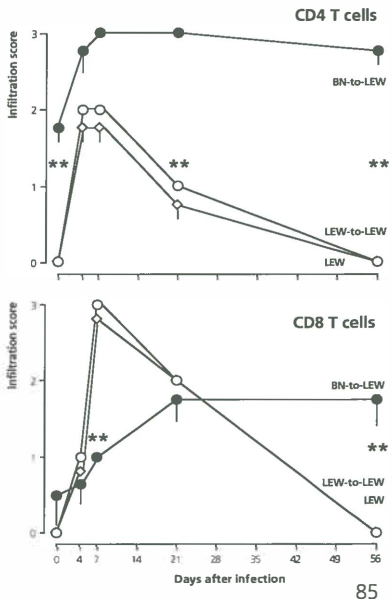
Figure 8. Infiltration of T cells.

Already before infection, CD4 positive and few CD8 cells were present in the allografts, and not in the other lungs.

CD4 T cells. After infection, CD4 cells increased sharply in all 3 groups. Their numbers remained high in the allografts whereas they declined to zero in the isografts and nontransplanted lungs.

CD8 T cells. The pace of infiltration of CD8 cells was remarkably slow in the allografts: they increased steadily to reach their maximum only by day 21, and remained present. In the isografts and nontransplanted lungs CD8 cells increased sharply after infection, with a peak on day 7, and declining thereafter.

(** = p<0.01)



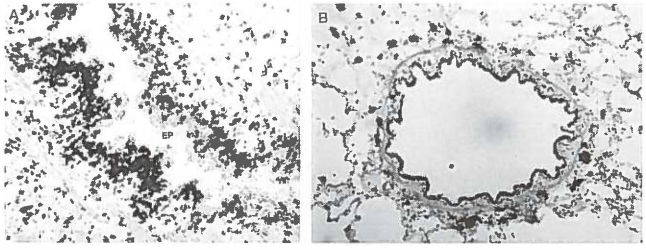


Figure 9. CD8 cells day 56 after infection

A. Allografts
B. Isografts

Magnification x65.
EP is epithelium.

IL2-receptor

The IL2-receptor, T cell activation marker was not present in the airways of the non-infected rats of all 3 groups. Within 7 days after viral infection, IL2-receptor-positive cell numbers around the airways reached a peak in all three groups, but this peak was higher and lasted longer (till day 21) in the allogeneically transplanted lungs.

B cells

Figure 10.

Already in noninfected allogeneic transplants B cells were present around the bronchioles, whereas they were absent in the syngeneically transplanted and non-transplanted lungs. After infection the number of B cells did not change at all in the allogeneically transplanted lungs during the whole observation period. In contrast, in the syngeneically transplanted and nontransplanted lungs B cell numbers around the bronchioles started to rise at day 4 after infection, reaching a peak on day 21 after infection and dropping afterwards.

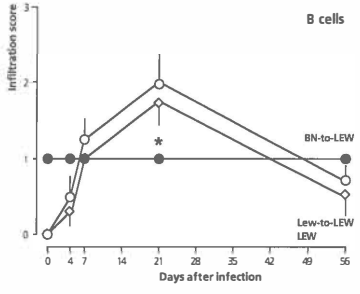


Figure 10. Infiltration of B cells.

Already before infection B cells were present peribronchiolly in the allografts and not in the other lungs. After infection B cells did not increase in the allografts during the whole observation period. In contrast, in the isografts and nontransplanted lungs B cells infiltrated around the airways, reaching a peak on day 21 and dropping afterwards.

(* = $p<0.05$)

Control lungs in the allogeneically transplanted group

Chronic rejection alone did not cause a detectable increase in histological changes and inflammatory and immune parameters between 6 and 8 months after transplantation: the results of the non-infected allogeneically transplanted lungs investigated at 6 months and 56 days later (8 months) were similar.

Infection alone, in the contralateral nontransplanted right lung of the allogeneically transplanted rats had a similar course as in syngeneically transplanted and non-transplanted rats.

Discussion

This study shows that a mild respiratory viral infection induces an abnormally pronounced inflammatory response in the bronchioles of long-term surviving allogeneically transplanted rat lungs. The viral infection aggravated the minimal inflammatory response that was already present in the airways of the noninfected allografts as a result of chronic rejection. Also, the immune response after infection was highly abnormal in the allogeneically transplanted lungs: it started slow, but then persisted till the end of the observation period. In the syngeneically transplanted and nontransplanted lungs the inflammatory response induced by infection was weaker while the immune response started swifter after infection; both responses were completely resolved 56 days after infection. The aberrant inflammatory response in the allogeneically transplanted lungs corresponded with early and severe epithelial damage in the bronchioles in these lungs, finally progressing into BO.

Chronic rejection in noninfected allogeneic rat lung transplants does not induce inflammatory and immune responses severe enough to cause BO. In the bronchioles of the lung allografts with chronic rejection we observed a low-grade response, as can be concluded from the presence of ED1 positive macrophages and CD4 positive T cells, but without signs of strong activation: as demonstrated by the virtual absence of inflammatory ED2 positive macrophages, O_2^--producing PMNs, MHC class II expression on the epithelium

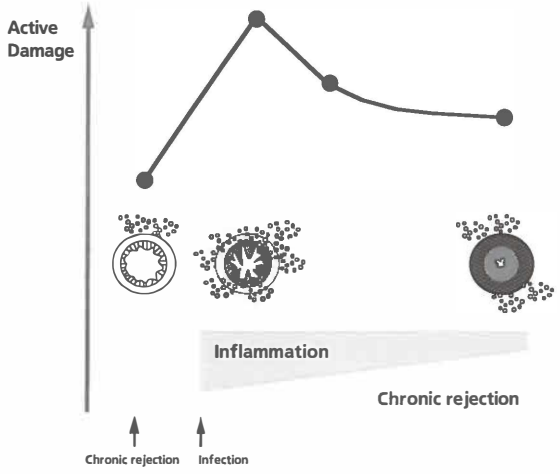
and expression of IL2-receptor on T cells. This response in the non-infected lung allografts was stable during the 56 days observation period and was not accompanied by damage of the bronchioles. This seems to be in contradiction with findings in a previous study where we observed late airway changes in long-term surviving rat lung allografts in association with a similar chronic rejection process (15). However, the focal airway changes described in that study were restricted to the large airways where MHC class II antigens were expressed locally on the bronchial epithelium. These airway changes were, besides MHC class II expression, accompanied by submucosal recipient dendritic and lymphocytic infiltrates. In this study, where we focussed on the bronchioles, the expression of MHC class II antigens on the bronchiolar epithelium and recipient dendritic cell aggragates were absent. We assume that the higher number of recipient dendritic cells in the large airways contribute to the difference in airway changes between the large airways and bronchioles in non-infected rat lung allografts (21). Previously it has been mentioned that dendritic cells take up alloantigens from the graft, process them and present the antigens to recipient T cells (22); in this way recipient dendritic cells have been shown to trigger rejection of skin grafts (23). Obviously, this process is not strong enough to cause BO-like airway damage in the bronchioles.

Likewise, isolated Sendai viral infection in normal lungs and syngeneically transplanted lungs does not result in BO-like airway damage. The viral infection did initiate an inflammatory response, with the immediate infiltration of ED1 positive macrophages, inflammatory ED2 positive macrophages, O_2^- producing PMNs, NK cells and expression of MHC class II antigens on the bronchial epithelium, but this response is completely resolved within a few weeks after infection. Simultaneously with the inflammatory response an immune response was initiated in these lungs as can be concluded from the infiltration of CD4 and CD8 positive T cells, associated with expression of the IL2-receptor on these T cells and infiltration of B cells. After a peak on day 7 all

parameters of the inflammatory and immune responses decreased and were completely absent at the end of the observation period. The associated airway changes were mild without necrosis of the epithelium and were completely resolved by day 56 after infection. Similar inflammatory responses with minimal histopathological airway damage have been observed in other respiratory viral infections (20, 24, 25). Although these responses induce airway changes, they are part of the normal host defense, important for the clearance of the virus from the airways and for the induction of repair mechanisms. After the virus has been cleared from the airways the trigger for the response is eliminated and it will extinguish without leaving residual damage. This sequence has been shown in studies in normal mice where after respiratory viral infection the extinction of the inflammatory and immune responses corresponded with clearance of the virus from the bronchiolar epithelium and disappearance of histopathological damage (20, 24). Similarly, we found in a previously study that after infection with Sendai virus in normal rat lungs the virus was only shortly present (18) which correlates with the limited responses in these lungs as observed in the present study. So, in normal lungs short, although intense, inflammatory and immune responses do not lead to permanent airway damage.

In case chronic rejection and a respiratory viral infection occur simultaneously, preexisting changes are aggravated, resulting in severe and persisting inflammatory and immune responses in the bronchioles. In time two phases can be distinguished in these allografts: the early phase, till day 21 after infection, when the inflammatory response predominates, and the late phase, from day 21, when the immune response becomes prominent. In the early phase of infection an intense inflammatory response causes severe damage of the bronchioles: on day 7 after infection the epithelium of most of the bronchioles was destroyed, which coincided with high numbers of O_2^- producing PMNs. Thereafter, in the late phase, the intensity of the inflammatory response declined, but remained active as

charactarized by persisting ED1- and ED2 -positive macrophages and PMNs. Of interest is our finding that the immune response showed an inverse relation with the inflammatory response and became more prominent in the late phase after infection, in particular the influx of CD8-positive cells was remarkebly delayed. However, once they were high the number of both CD4- and CD8-positive cells remained so, predominating the response in the late phase after infection. These intense inflammatory and immune responses finally resulted in BO-like airway damage.



The possible synergistic action of inflammatory and immune responses that causes bronchiolitis obliterans after viral infection in lung allografts with chronic rejection. Two phases can be distinguished: early after infection inflammation predominates, whereas in the second phase the immune response, most likely enhancedd chronic rejection, is most prominent.

Two conceivable causes of the aberrant inflammatory and immune responses have been suggested after viral infection in lung allografts with chronic rejection: an impaired defense against the virus and second, stimulation of the rejection process by the viral infection (15). We assume that it is the combination of these two causes that induce the aberrant inflammatory and immune responses in infected rat lung allografts. First, the early and aberrant inflammatory response might be caused by an impaired host defense against the virus

in the lung allograft. In a separate study we have demonstrated that in lung allografts the local and systemic antibody response against Sendai virus is impaired, resulting in prolonged presence of the virus in the airways (18). Similarly, in the present study the number of B cells around the bronchioles did not increase in the allogeneically transplanted lungs after infection, in contrast to B cell numbers in the normal lungs. Next to a defect in the humoral response, also the cellular immune response was different in the allogeneically transplanted lungs. In the normal lungs the infiltration of NK and CD8-positive cells was initiated immediately after infection. In the allogeneically transplanted lungs, however, theses responses were strikingly delayed. Several other studies, using different types of respiratory virusses, showed that the rapid infiltration of NK and CD8-positive cells is important for adequate clearance of the virus from the airways, once the epithelium is infected (24, 25, 26). This study suggests that in lung allografts, in addition to a defect in the local humoral response, the cellular response after a respiratory viral infection is impaired, thereby contributing to a reduced anti-viral defense.

Secondly, the aberrant inflammatory response in the early phase after infection might activate the rejection process, possibly seen as the immune response in the late phase after infection. Such an increase in alloreactivity has been demonstrated clinically in cardiac transplant recipients after active influenza vaccination (27) and in lung transplant recipients after CMV infection (8) and pneumocystis infection (17). From the present study we cannot conclude whether a similar increase in alloreactivity occurs after Sendai infection. Yet, it is tempting to assume that the infiltrates of CD4- and CD8-positive cells in the allogeneically transplanted lungs remaining from day 21 after infection are driven by an alloreactive process. For, these infiltrates persisted well beyond the time when the virus was eventually cleared from the allograft on day 21 (18) and such infiltrates were absent at the same time in the contralateral right non-transplanted lungs. However, it is questionable whether these potentially

alloreactive cells do cause tissue damage in the lung transplant as most of the epithelial damage was seen in the early phase after infection.

Since in this experimental study the inflammatory response seems to play a primary role in the induction of airway damage after lung transplantation it would be a logical step to suggest for clinical treatment to suppress the inflammatory response in any event, irrespective whether it is rejection or infection. This is supported by the clinical observation that without exact diagnosis, a deteriorating lung function in lung transplant recipients can be favorably improved by treatment with steroids (28), even in patients that later turn out to be infected. Similarly, a recent preliminary clinical study suggested that the oxygen free radical scavenger allopurinol might reduce the development of BO in lung transplant recipients with declining lung function (29). These findings in combination with the results from the present study warrant further investigations on the role of inflammation in the development of BO in lung transplants.

Conclusion

This study shows that in allogeneic rat lung transplants the combination of chronic rejection and a respiratory viral infection results in severe inflammatory and abnormal immune responses that induce BO. It is conceivable that also in patients simultaneous occurence of (chronic) rejection and infection will induce a stronger inflammatory response than their isolated occurence, which then easily could induce BO. This would explain the clinical feeling, if not observation, that a combination of (chronic) rejection and infection often preceeds the development of BO (10, 30). In this view, agressive suppression of the early inflammatory response after infection in lung allografts might play a dual role: preventing early and severe damage of the airways and second, preventing activation of rejection.

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3 Immune responses in infected rat lung transplants

The purpose of this chapter is to briefly review the defense of the normal lung against inhaled antigens, with an emphasize on the mechanisms responsible for the induction of local immune responses in the lung against these intrapulmonary antigens.

The lungs are the only organs in the body that are continuously in open contact with the outside air. The surface area of lung tissue in contact with air is approximately that of the size of a tennis court (1). This enormous air-lung tissue interface permits easy uptake of oxygen from the air and removal of carbondioxide from the body: the process called gas-exchange. Together with air, however, potential pathogens and antigens are inhaled that can damage the lung. In order to eliminate these antigens the lung is equipped with an extense defense system. This defense system is designed to prevent injury, infection, and invasion of the lung tissue (2,3,4) The defense system of the normal lung can be divided into antigen non-specific mechanisms and antigen-specific humoral and cellular immune mechanisms.

Nonspecific defense

In the large airways inhaled antigens are cleared mechanically by mucociliary transport and nervous reflexes causing cough (5). At the same time contractions of smooth muscle cells in the bronchial walls decrease the diameter of the airways thereby preventing spreading of antigen into the periphery of the lung. When antigens reach the alveoli the alveolar macrophage is of prime importance for antigen removal (6). The majority of these antigen-containing macrophages are removed from the lung by the mucociliary escalator. Other macrophages, however, appear to gain entry to the lung tissue where they migrate via lymphatics to the lung-associated lymphnodes, where they induce antigen-specific immune responses against the phagocytised antigens (7, 8). The latter fate of intrapulmonary antigens will be discussed in more details in the next paragraphs. The nonspecific defense mechanisms operate continuously at the epithelial surface of

the lung tissue to maintain sterility of the lung and they do not necessarily depend on the immune status of the host (3). Although these mechanisms usually function adequately they can be overwhelmed by inhalation of massive numbers of antigens or highly virulent infectious agents. When these harmful antigens break through the nonspecific epithelial defenses the antigen-specific immune system of the lung is triggered as a second line of defense.

Antigen-specific defense

Immune responses comprise the antigen-specific mechanisms of host defense forming the so-called mucosal immunity of the lung. These immune responses can be divided into two major effector systems: humoral or antibody-mediated immunity and cell mediated immunity (9). The immune responses of both types are initiated by antigenic stimulation and are effected by B- and T lymphocytes. The soluble end-products of B lymphocytes are antibodies that serve to enhance phagocytosis, promote bacterial killing and neutralize virusses. The T lymphocyte effectors may either be cytotoxic for virally infected cells or they may secrete cytokines like IL2,-3,-4 and 5, that enhance humoral responses and inflammatory responses effected by macrophages and polymorphonuclear cells.

Both B and T lymphocyte mediated immune responses are induced in the immune system that can be found throughout the lung. The degree of structural organization of the immune system increases progressively from the distal alveolar spaces to the proximal large airways. In general, the immune system of the lung can be divided into three important components: 1/ immunocompetent cells in the respiratory epithelium and lung parenchyma, 2/ lymphoid structures associated with the bronchial epithelium (bronchus-associated lymphoid tissue or BALT), and 3/ lymphnodes draining the respiratory system via afferent lymphatics. Antigens deposited in the lung may be eliminated by either of these three immune systems, depending of the type of antigens and the site of deposition.

Immunocompetent cells in respiratory epithelium and lung parenchyma

In the normal lung isolated lymphoid cells can be found throughout the respiratory tract. They are present in the respiratory epithelium, in the submucosa and in the alveolar septa. Recent studies have demonstrated that they are predominantly T cells and that the amount of these T cells in the lung tissue is comparable to that in the peripheral blood pool (10). These, predominant CD8-positive suppressor/ cytotoxic, T cells are suggested to play an important role in the suppresion of immune responses in the lung to avoid allergic sensitization against nonpathogenic antigens (11). A more detailed discussion about their function and role in this regard is beyond the scope of this thesis and can be found elsewhere (7, 11). Together with these T cells, the epithelium and submucosa of the airways contain large numbers of dendritic cells (12). Interestingly, they form a dense network in the respiratory epithelium with a great resemblance the Langerhans' network in the skin (13). Dendritic cells have a strong antigen presenting capability and they are believed to be the most important antigen presenting cells in the lungs (14).

The vast majority of cells in the alveolar space are macrophages and in humans about 10% of the total cells are lymphocytes (15). Antigens in the alveolar spaces can by phagocytised by these alveolar macrophages and are subsequently removed from the lung by the mucuciliary escalator. Also, the antigen-containing macrophages can be taken up in the BALT or by lymphatics and transported to the lung-associated lymphnodes where they induce immune responses (9, 16, 17). Furthermore, the alveolar macrophage plays an important role in the regulation of the immune response in the lung tissue (7, 18).

The local immune system in the lung: bronchus-associated lymphoid tissue

After respiratory infection, specific immunoglobulins against the infecting organism appear at the epithelial surfaces (19). These antibodies can be IgA, IgG or IgM and it is now generally assumed that this antibody production takes place locally in the lung (20, 21). In particular IgA is important on mucosal surfaces, where it plays a major role in resistance to infection by neutralizing virus and preventing the adherence of bacteria to mucosal surfaces (22, 23). For example, immunity to influenza is related to the titer of IgA antibody in nasal or bronchial secretions (24, 25). A correlation between the presence of secretory IgA and protection against viral infection has also been shown for several other viruses, including parainfluenza type I, rhinovirus, poliovirus and respiratory syncytial virus (25, 26). The role of IgA in the defense against respiratory viral infections is further emphasized by the high incidence of these infections in IgA-deficient patients (27, 28). Most importantly, it appears that the production of secretory IgA is a local phenomenon occuring at mucosal sites directly stimulated by viral antigen. For example, aerosol vaccination with influenza virus stimulates only the production of nasal and bronchial antibody (24, 29).

These local antibody production in the lung takes place in the BALT in most mammals. The first observation of BALT in in the lung was made by Klein in 1875 (30). However, it was only 100 years later, in 1973, that Bienenstock gave a detailed description of this lymphoid tissue (31). It was named bronchus-associated tissue or BALT because of its resemblance to Peyers' patches (the gut-associated lymphoid tissue or GALT). The prominence and quantity of BALT varies between species (32, 33), being markedly prominent in birds, well developed in rabbits and rats, and less easily detected in dogs and humans. Some authors even suggest that the BALT is not a constituent part of the human lung (33, 34). Nevertheless, in man, diffuse collections of sub-epithelial lymphocytes can be found which become more dense after antigenic stimulation:

clinical studies demonstrated the existence of pronounced BALT in patients with respiratory infections (35, 36). Although BALT may be formed anywhere within the bronchial mucosa, it is found especially at sites where inhaled antigens are present in highest concentrations, i.e., at the airway bifurcations (32, 33, 37, 38). Here, the BALT is located between a bronchial artery and the bronchus (32, 33) (Figure 1).

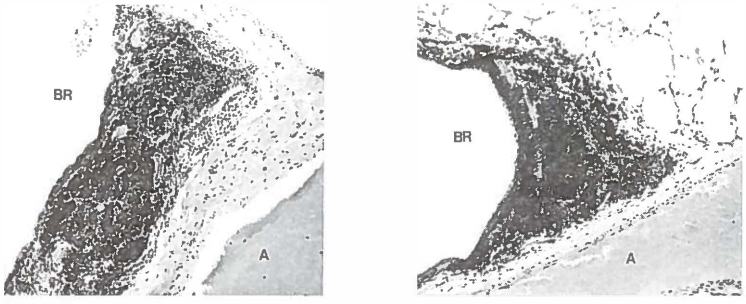


Figure 1. BALT in rat lungs.

This figure shows 2 different types of BALT that can be found in normal, noninfected rat lungs. BALT consists of a dense collection of lymphoid cells directly situated under the bronchial epithelium. BALT is usually located between a bronchus (BR) and a bronchial artery (A).

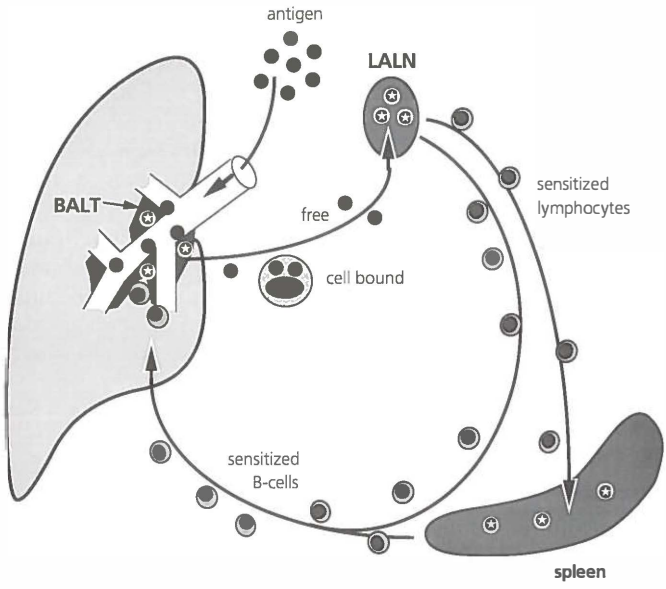
The BALT consists of a dense collection of lymphoid cells situated directly under the bronchial epithelium. The epithelium overlying the BALT is different from normal bronchial epithelium and has the characteristics of lymphoepithelium. It consists of flattened non-ciliated epithelial cells, infiltrated by lymphocytes. The lymphoid cells in the BALT are predominantly IgA , IgG and IgM-containing B cells (32, 39). T cells are less numerous and account for about 30 to 40 % of the cells in the BALT. In contrast to lymphnodes, no distinct B and T cell areas are found in the BALT of most species (32, 40). In addition non-lymphoid cells like interdigitating cells, dendritic cells and macrophages are

present in the BALT (40).
The BALT contains both arterioles and venules and has an extensive capillary network. In addition high endothelial venules (HEV) are found throughout the BALT (32, 42). The BALT has no afferent lymphatics and recent studies indicate that circulating lymphocytes can only enter through these HEV (43), providing a pathway for the traffic of circulating lymphocytes into and through the BALT. Lymphocytes can leave the the BALT via efferent lymphvessels and drain into a the systemic circulation or into lung-associated lymphnodes (32, 39).

Although the exact function of BALT is still unclear, it is evident that BALT plays an important role in antigen sampling from the airways and local production of antibodies, especially IgA, against these antigens. Secondly, antigens that are taken up in the BALT are found to accumulate in the lung-associated lymphnodes (LALN), where induce immune responses (fig 2).
Antigens administered in the lung are transported through the lymphoepithelium into the BALT (32). It has been shown that different types of antigen like ferritin, horseradish peroxidase, viruses and bacteria adhere to and penetrate the lymfoepithelium via specialized M cells (33, 44). Once taken up in the BALT these antigens are processed by dendritic cells and presented to the lymphoid cells. This process induces a plasmacellular reaction resulting in antibody-containing cells. Simultaneously, lymphocytes are recruited from the circulation and involved in the immune response in the BALT. As a result of this strong local immune response the size of the BALT can increase immensely (45). The antibodies are secreted into the airway lumen where they can exert their protective role. As mentioned above, antigens that are taken up in the BALT are also found to accumulate in the lung-associated lymphnodes (LALN). Apparently they are transported through the lymphvessels that drain the BALT. From these LALN, sensitized lymphocytes also migrate to the spleen. In response to the antigen, large numbers of antibody forming cells are generated in the LALN and the spleen (32),

which recirculate preferentially to the site of antigenic stimulation in the lung. Figure 2. summarizes the sequence of antigen deposition in the lung and its subsequent fate.

Figure 2.



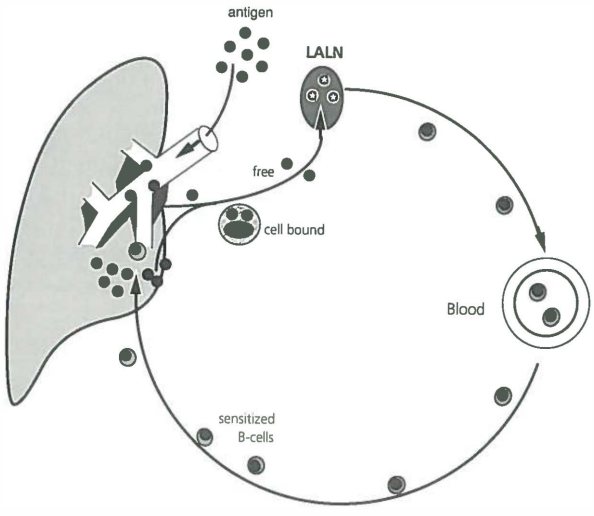
In addition, it has been suggested that the BALT and the lung are part of a common mucosal immune system of the body (46, 47). It has been shown that precursor IgA-producing cells migrating from the gut immune system do not only home preferentially to the lamina propria of the gut, but also to the respiratory tract (45, 46). It is, however, uncertain whether the opposite route, i.e., from the BALT and the lung to the gut is also a preferential route of migrating lymphocytes (32, 45).

Lymphnodes draining the lung

In the lung-associated lymphnodes (LALN) antibody responses against intrapulmonary particulate antigens are generated (9). Several lung-associated lymphnodes in the thoracic cavity receive lymphatic drainage from the lung and each LALN receives lymphatic drainage from a specific lung lobe or lobes (48, 49, 50).

Although it has been extensively demonstrated that the LALN are the main site of immune responses after immunization with particulate antigen (9) (Fig 3.), the mechanisms by which antigen leaves the lung and reaches the LALN is not completely understood. As mentioned before, most particulate antigens deposited in the airways and alveolar spaces are cleared by phagocytosis by alveolar macrophages (6, 7), which then transport the foreign material over the mucociliary escalator out of the lung. In addition, however, macrophages containing antigen are transported to the LALN that allows antigen to be presented to the lymphoid cells in the LALN (8). At present it is unclear whether all antigens are taken up in the BALT and then transported to the LALN, or that antigens are also be taken up at other sites of the lung. After induction of the immune response in the LALN, specific antibodies are released into the systemic

Figure 3.



circulation, and these antibodies return to the site in the lung where the antigen is present (8, 50). The sequence of antigen deposition in the lung and subsequent antibody production is summarized in fig 3.

Lung transplantation and lung defense

Inhalation of antigens in the normal lung leads to non-specific and and antigen-specific defense mechanisms that, in most cases, protect the lung from infection and injury. Under certain pathologic conditions the defense systems of the lung fail, putting the lung at risk for infections. Lung transplantation seems to be one of these conditions.

As mentioned in chapter 1.2 intrapulmonary infections are a major complication after lung transplantation, causing high morbidity and mortality. In addition we have found in chapter 2 that respiratory viral infections aggravated the airway damage caused by chronic rejection in rat lung transplants, which was attributed to aberrant inflammatory responses. These data suggest that the normal lung defense is impaired in transplanted lungs. Therefore, we investigate in this chapter whether antigen-specific immune responses against intrapulmonary antigens are disturbed in the transplanted lung. We will specifically investigate the route from deposition of particulate antigen in the transplanted lung to antibody response in the hilar lymphnodes with intrapulmonary injected sheep red blood cells in chapter 3.2. The influence of interruption of lymphatics by the transplantation procedure on the antibody response in the LALN will be investigated in chapter 3.3. In chapter 3.4 we will investigate the local antibody production in the BALT of the transplanted lung after respiratory viral infection. In chapter 3.5 we specifically investigate structural aspects of the BALT in transplanted and nontransplanted lungs.

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3.2 Reduced antibody responses after immunization in rat lung transplants

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J Heart Lung Transplant 1992; 11: 222 (abstract)

3.2 Reduced antibody responses in lung rat transplants

Abstract

Pulmonary infections occur so frequently in recipients of (combined heart and) lung transplants that it has been suggested that the function of the defense system in lung transplants is impaired. Therefore we investigated in rats whether antibody responses against intrapulmonary antigens were impaired at various time-points after transplantation. Antibody responses were induced in lungs of 4 experimental groups: group 1: normal lungs (LEW), group 2: hilar stripped (sham operated) lungs (LEW), group 3 syngeneic lung transplants (LEW-to-LEW) and group 4: allogeneic lung transplants (BN-to-LEW). The operations were performed on the left lungs. All rats (including those with normal lungs) were treated with cyclosporine on days 2 and 3 after operation, which treatment is adequate to induce permanent graft acceptance of the allografts. Rats were immunized 7, 10, 14, 21 and 28 days and 6 months after operation with sheep red blood cells, injected selectively into the bronchus of the left lung. The resulting serum antibody titers were detected with a hemolysis assay.

After immunization on day 7 no antibody responses could be detected in all hilar stripped and transplanted rats, whereas responses were normal in two allografted rats immunized in the non-transplanted right lung. After immunization on day 14, responses had returned to normal in hilar stripped rats, whereas they were still impaired in the transplanted rats. After immunization on day 28, responses were almost normal in all rats and remained so up till 6 months after transplantation.

We conclude that antibody responses in lung grafts are impaired early after lung transplantation. The degree of impairment, being less after hilar stripping than after transplantation, depends mainly on the extent of the surgical trauma. These findings may explain why pulmonary infections occur so frequently in the early period after lung transplantation.

Introduction

Infections are the major cause of early and late morbidity and mortality after heart-lung and lung transplantation (1,2). Lung allograft recipients with early pulmonary infections have an increased mortality rate compared with recipients without early infections. A recent study reported a one year mortality rate of 65 % for patients with early infections versus 33 % for patients without early infections (3)

The incidence of pulmonary infections is much higher after lung transplantation than after heart transplantation (4,5). Seventy percent of the infections occur in the transplanted lung as the primary site (2) In fact, pulmonary infections occur so frequently after lung transplantation that

it has been suggested that the defense of the lung against pathogens is impaired after lung transplantation (1,2,3).

The defense system of the normal lung can be divided into a mechanical part: - bronchial smooth muscle contraction, cough and transport via the mucociliary escalator - and a specific part, including immune reactions (7). To induce an antibody response against antigens deposited in the lung, these antigens are to be phagocytosed by pulmonary macrophages and polymorphnuclear leukocytes and transported via the pulmonary lymphatic vessels to the draining lung-associated lymphnodes (LALN). In these lymphnodes antibody-forming cells are generated and antigen-specific antibodies are produced and released into the blood. It has been shown that only if antigens in the lung are transported to LALN, an appropriate immune response can be generated (8,13,14,15). Clinical and animal studies sofar solely focussed on the mechanical part of lung defense system, e.g. mucocilliary transport and disruption of lymphatics after lung transplantation (6,9). As a result, no information is available about the capability of lung recipients to generate an appropriate immune response against intrapulmonary antigens after lung transplantation.

Since pulmonary infections are most frequent early after transplantation, we hypothesized that the antibody response against antigens is impaired in this period. Therefore, we investigated the systemic antibody response against sheep red blood cells (SRBC) after intrapulmonary immunization at different intervals after allogeneic lung transplantation in rats. To analyse the influence of the transplantation procedure and the interruption of lymphatics on the antibody response, syngeneically transplanted lungs and hilar stripped lungs served as controls.

Materials and methods

Experimental design

In this study antibody responses were induced in lungs of four experimental groups: group 1: normal lungs, group 2: hilar stripped lungs (HS), group 3: syngeneic lung transplants (LEW-to-LEW) and group 4: allogeneic lung transplants (BN-to-LEW). All rats were immunosuppressed with CsA injected on days 2 and 3 after transplantation or hilar stripping; the normal rats received CsA for two days, too.

Antibody responses were induced selectively in the left lung with 10^9 SRBC in 100 µL saline, at 7, 10, 14, 21 and 28 days and 6 months after transplantation or hilar stripping. After immunization serum antibody titers were followed during 1 month.

Table 1 summarises the immunization schedule and the number of rats in the experimental groups. Normal, i.e. non-operated but CsA treated rats of the same age were immunized as controls.
Because CsA treatment was found not to affect antibody responses in these normal animals, data of day 7 normal rats were used as controls on days 14, 21 an 28 (see figs 4, 5 and 6).

Table 1.

Group	Immunization schedule					
	day 7	day 10	day 14	day 21	day 28	6 months
Normal LEW	5	5	4	ND	ND	4
HS LEW	4	4	4	ND	4	ND
LEW-to-LEW	5	5	4	4	4	4
BN-to-LEW	7@	4	4	4	4	4

Immunization schedule after hilar stripping or transplantation and number of rats immunized on each time-point.

All rats received CsA for 2 days.
Normal rats had no operation on day 0.
ND is not done.
@ Two rats were immunized in the right, nontransplanted, lung

Rats

Young adult, male, specific pathogen-free LEW ($RT1^l$) and BN ($RT1^n$) rats, weighing 250-350 grams were obtained from Zentral-Institut für Versuchstiere, Hannover, Germany. All animals received humane care complying the Dutch regulations and law.

Hilar striping and Lung transplantation

The technique of hilar stripping in rats has previously been described (10) In brief, after thoracotomy in the left fifth intercostal space,all surrounding tissues were dissected meticulously from the left main stem bronchus, pulmonary artery and vein, resulting in the complete disruption of the hilar lymphatics, the bronchial arteries and the branches of the vagal nerve to the left lung.

Left lung grafts were orthotopically transplanted in the thorax, according to the improved technique of Marck and Prop (11,12). Briefly, the donor lung was dissected and its vascular bed flushed with cold saline. The recipient's left lung was removed and replaced with the donor lung; the pulmonary vein and artery were anastomosed before the bronchus.

The function of hilar stripped and transplanted lungs was monitored by chest roentgenography. All chest roentgenograms showed normal appearance of the lung at the day of immunization.

Immuno-suppression

All rats received cyclosporine (provided by Sandoz Pharmaceuticals Corporation, Basel, Switzerland), dissolved in olive oil and injected intramuscularly in a dosage of 25 mg/kg. body weight on days 2 and 3 after operation, both after lung transplantation and hilar stripping. This treatment is adequate to induce permanent graft acceptance of the allografts. Normal control animals also received CsA for 2 days.

3.2 Reduced antibody responses in lung rat transplants

Immunization antigen

In this study all rats were immunized with sheep red blood cells (SRBC). As demonstrated in other studies SRBC evoke specific antibody responses in rats of which the antibodies can be detected in peripheral blood (13,14). Pilot studies showed that a dosage of 10^9 SRBC in 100 µL saline, injected intrabronchially, evokes a clearly detectable antibody response in the blood of normal LEW rats.

Throughout this study, SRBC were obtained from the same animal

Intrabronchial administration of SRBC

To investigate the antibody response in the left transplanted or hilar stripped lung SRBC were selectively administered in the left lung. To achieve this selective administration we developed a method for selective intrabronchial administration of antigens in the rat. Rats were anaesthetised with 4% halothane in a gas mixture of 30% oxygen and 70% nitrous oxide and intubated with a tracheal cannula as has been described before (12). After intubation, a thin curved catheter, with a smaller diameter and a metal coated tip, was introduced through the intratracheal tube into the lower end of the left bronchus. The correct location of the intrabronchial catheter was confirmed under fluoroscopic control. Then 100 µL of saline with 10^9 SRBC was instilled via this catheter in the left lung. Immediately after instillation the intratracheal tube and catheter were removed.

Antibody titers

At intervals after immunization (4, 7, 10, 14 ,21 and 28 days) blood samples for antibody titer measurements were obtained by retro-orbital puncture. The total amount of antibodies (IgM plus IgG) against SRBC in the peripheral blood was detected with a hemolysis assay. Titers are expressed as the 2log of the last dilution showing hemolysis.

Statistical analysis

Mean titers and standard deviations of the hemolysis assays were calculated on the basis of the 2log titers of the individual titers. Antibody titers in the different experimental groups were compared with the Mann Whitney U test for unpaired observations. Differences were considered to be significant if the p-values were < 0.05. The calculations were performed with the statistical software package Statview™ for the Apple MacIntosh computer.

Results

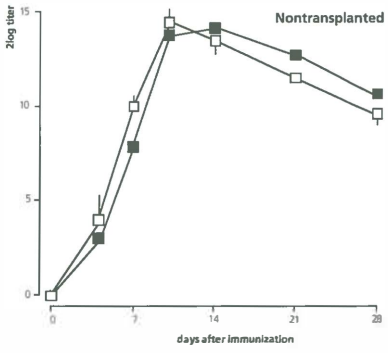
After immunization of *normal LEW rats* antibodies appeared in the blood on day 4 after immunization, reaching a peak on day 10. After day 10 the antibody titers fell gradually (fig 1). CsA did not affect the antibody response against SRBC instilled on day 7 or 10 as normal rats treated with CsA on days 2 and 3 showed similar responses as untreated normal rats (fig 1).

Figure 1. Normal rats.

Antibody response in the blood after immunization with 10^9 SRBC in the left lung of norma l (open squares) and CsA-treated rats (closed squares).

Serum antibody titers were followed for 1 month. Anti-SRBC antibody titers in the blood are expressed as the 2log of the last dilution showing hemolysis. Data are mean ± SD.

Antibodies appeared in the blood on Day 4 after immunization, an they reached a peak on Day 10. Normal and CsA-treated animals showed identical responses. This normal curve will be used in the following figures as normal reference.



3.2 Reduced antibody responses in lung rat transplants

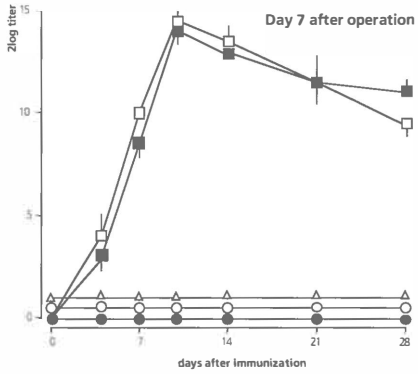


Figure 2. Day 14 after operation.

Antibody responses were absent in all operated groups HS (open triangles), LEW-to-LEW (open circles) and BN-to-LEW (closed circles) transplantation groups. In contrast, two animals in the BN-to-LEW group that were immunized in the nontransplanted right lung (closed squares) showed normal responses.

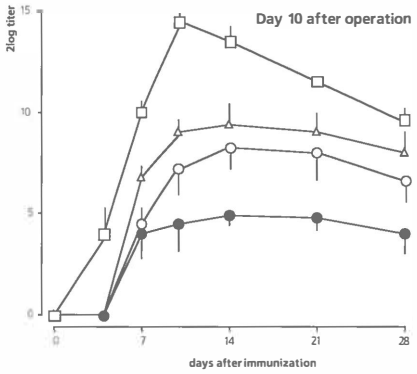


Figure 3. Day 10 after operation.

Antibody responses became detectable in all operated groups, but they were signficantly lower than in normal animals at corresponding time-points. The onset and peak of the immune response were delayed in all operated groups.
HS (open triangles): $p<0.05$.
LEW-to-LEW (open circles) and BN-to-LEW (closed circles): $p<0.01$.

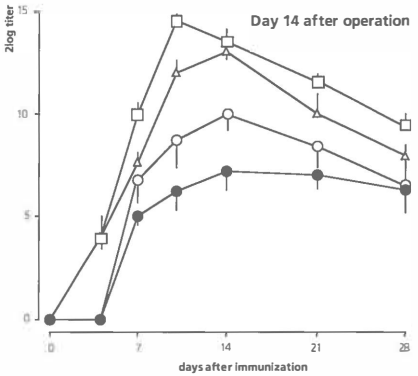


Figure 4. Day 14 after operation.

Antibody responses in the HS group (open triangles) were normal, whereas responses were still significantly lower in the LEW-to-LEW (open circles) and BN-to-LEW (closed circles) groups ($p<0.05$).

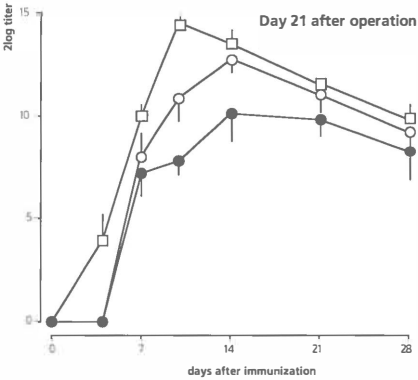


Figure 5. Day 21 after operation.

Antibody responses in the LEW-to-LEW group (open circles) reached normal values, but the onset and peak were delayed compared with responses in the normal rats. Responses in the BN-to-LEW group (closed circles) were still significantly lower than in normal rats ($p<0.05$).

After immunization in the left lung on *day 7* after hilar stripping and transplantation antibody responses were absent in all rats in the HS, LEW-to-LEW and BN-to-LEW groups. No antibodies were detectable during the whole observation period. In contrast, 2 animals in the BN-to-LEW group that were immunized in the right lung showed normal antibody responses (fig 2).

After immunization on *day 10* after operation antibody responses became detectable in all operated groups, but were significantly lower than in normal animals (HS $p<0.05$, LEW-to-LEW and BN-to-LEW $p<0.01$ compared to normal values on corresponding time-points) (fig 3). The appearance of antibodies was delayed in all groups and first detectable on day 7 after immunization. Furthermore, the antibody titer reached a peak more slowly, on day 14 after immunization.
After immunization on *day 14* after operation antibody responses in the HS groups were normal, whereas responses were still significantly impaired in the LEW-to-LEW and BN-to-LEW transplanted groups ($p<0.05$ compared to normal values on corresponding time-points). The onset and peak of the antibody response in these transplanted groups were still delayed (fig 4).

After immunization on *day 21* after operation antibody responses in the LEW-to-LEW group reached normal values, but the onset and peak were delayed compared to responses in normal rats. Antibody responses in the BN-to-LEW transplanted group were still significantly lower than in normal rats ($p<0.05$ compared to normal values on corresponding time-points) (fig 5).

After immunization on *day 28* antibody responses in the BN-to-LEW transplanted group reached normal values like in the LEW-to-LEW group. But, the onset and peak remained delayed in both transplanted groups compared to responses in the normal and HS groups (fig 6).

After immunization *6 months* after operation antibody responses in the LEW-to-LEW and BN-to-LEW transplanted groups were similar as in normal LEW rats of the same age (fig 7).

3.2 Reduced antibody responses in lung rat transplants

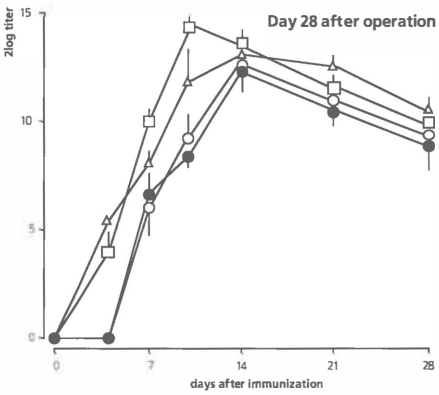


Figure 6. Day 28 after operation.

Antibody responses both in the LEW-to-LEW (open circles) and BN-to-LEW (closed circles) transplanted groups reached normal values. However, the onset and peak remained delayed in both transplanted groups compared with responses in the normal (open squares) and HS (open triangles) groups.

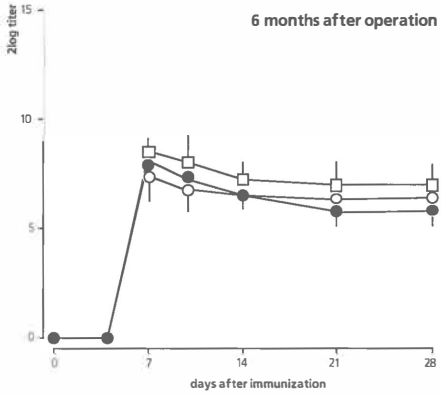


Figure 7. 6 months after operation.

Antibody responses in the LEW-to-LEW (open circles) and BN-to-LEW (closed circles) groups were similar to those in normal rats (open squares) of the same age.

Discussion

This study has demonstrated that the systemic antibody response against antigens applied in lung transplants is impaired in the early postoperative period after transplantation. Antibody responses returned to almost normal in 1 month after transplantation. However, the pattern of the antibody response remained delayed compared to the onset and peak in normal animals. Irrespective, of the kind of operation - hilar stripping, syngeneic or allogeneic lung transplantation - the antibody responses were equally impaired early after

operation, but they returned most slowly to normal values in allografted rats.

The impaired antibody response against SRBC in the hilar stripped, syngeneically and allogeneically transplanted lungs early after operation suggests a common factor in these groups that causes this impairment in the immune response. This factor has to be a local one related to the lung operated upon, as the response was normal in the animals immunized in the right lung on day 7. In a previous study we have demonstrated that when hilar lymphatics were interrupted by hilar stripping in rats, transport of particles from the lung to the LALN was disturbed and antibody responses were impaired (16). In the same study we demonstrated that lymphatics started to regenerate between 7 and 10 days after hilar stripping in parallel with a return of transport of particles to the LALN and normal antibody responses against SRBC. Our present study indicates that also after lung transplantation interruption of lymphatics is the most important cause of the impaired antibody responses early after transplantation: the pattern of impaired response and return of response largely corresponds with the interruption and regeneration of lymphatics we found previously after hilar stripping.

The different pace of restoration to normal antibody responses in the hilar stripped and transplanted rats might be explained by the differences in surgical trauma after hilar stripping and transplantation. Prop et al. previously demonstrated that damage to the operated lung is transient after hilar stripping and syngeneic lung transplantation in the rat, but that the recovery of the transplanted lungs was more slowly (10). In that study the more severe and prolonged edema in the transplanted lungs were found to lead to inflammatory reactions and fibrosis in the hilar region. In the present immunization experiments, the extensive edema early after lung transplantation might interfere with the uptake of antigens from the alveolar space in the lung and subsequent transport to the LALN. Later, at 28 days the hilar fibrosis in the transplanted lungs may persistingly hamper antigen transport and thus delay the antibody response.

In the present study, the allogeneically transplanted lungs recovered more slowly to normal antibody responses than the syngeneically transplanted lungs. A possible explanation for this is that besides the injury caused by the transplantation procedure itself, donor cells are to be replaced by recipient ones before antigen uptake and processing can proceed normally. It has been shown that dendritic cells in the allogeneic transplanted lung are replaced by recipient cells in about one month after transplantation (17). Similarly, it has also been demonstrated that in small bowel transplantation in rats the donor cells in the Peyer's patches, responsible for local antibody responses, are replaced by recipient cells by the end of the first month after transplantation (18). This suggests that during the first month after lung allotransplantation the immunologic mechanisms responsible for uptake and presentation of antigens from the lung are not functioning perfectly.

In long-term surviving lung transplants, both allogeneic and syngeneic, the antibody responses were similar as responses in normal rats of the same age, but they were lower than in young animals. These results show that in long-term surviving rat lung transplants a normal systemic antibody response is induced after immunization of the transplanted lung, although a defect in the response may be obscured by the aging effect.

In conclusion, this study shows that the transplantation procedure, and in particular the interruption of lymphatics, is the most likely cause of an impaired antibody response against antigens deposited in the transplanted lung in the early period after transplantation in rats. These findings are in concert with the clinical observatio that pulmonary infections have a high incidence in the first weeks after lung transplantation. Still, little is known about the local defense in the transplanted lung against pathogens, especially viral pathogens. In a recent study we found that viral infections cause more severe airway damage in allogeneic rat lung transplants than in syngeneic lung transplants and normal rats of the same age (19). Possibly the function of the local defense

system is impaired because of a lack of bronchus-associated lymphoid tissue in these allografted lungs (20, 21).

Our findings emphasize the importance of careful antibiotic measures during diagnostic interventions (e.g., bronchoalveolar lavage and transbronchial biopsies), which are potential sources of pathogens in the early postoperative period after lung transplantation.

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3.3 Influence of interrupted pulmonary lymphdrainage on antibody responses in hilar-stripped lungs

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3.3 Pulmonary lymphdrainage in hilar stripped lungs

Abstract

Lung transplantation interrupts hilar lymphatics. This may have an impact on immune responses to antigens entering the lung because the antigens cannot reach the lung-associated lymph nodes where the immune response is generated. We investigated the interruption and regeneration of lymphatics and the influence of this on antibody responses after hilar stripping in rats in three experiments: 1. visual detection of regenerated hilar lymphatics by chromolymphography, 2. observation of transport of carbon particles from the lung to the lung-associated lymph nodes and 3. assessment of antibody responses after lung immunization with sheep red blood cells.

The results showed that hilar lymphatics were interrupted by hilar stripping and regenerated from day 7 after operation. Transport of particles to the lung-associated lymph nodes was blocked during the first week after operation but returned to normal values thereafter. Serum antibody titers were absent or low in the rats immunized on days 7 and 10 after hilar stripping, subsequently antibody responses gradually recovered in one month. We conclude that antibody responses to antigens in hilar stripped lungs are impaired as long as the antigens cannot be transported through lymphatics from the lung to the lung-associated lymph nodes.

These findings can explain in part why pulmonary infections occur so frequently in the initial weeks after lung transplantation.

Introduction

After lung immunization, pulmonary macrophages and polymorphonuclear leukocytes can phagocytize particulate antigens deposited in the lung and transport the antigens via pulmonary lymphatics to the draining lung-associated lymph nodes (LALN). In these lymph nodes antibody-forming cells are generated and antigen-specific antibodies are produced. It has been shown that only if antigens in the lung are transported to LALN, an appropriate immune response can be induced (1 2 3). Lung transplantation causes interruption of the hilar lymphatics that form the connection between the lung and LALN. This interruption might have an impact on the production of appropriate antibody responses to antigens in the lung.

To investigate the influence of lymphatic interruption on antibody responses, we used in this study the method of hilar stripping of the lung, by which hilar lymphatics were interrupted as in lung transplantation.This method avoids other factors than lymphatic interruption that might impair immune responses after lung transplantation, such as graft ischemia and immunosuppression. We investigated in hilar stripped rats the following questions: whether hilar lymphatics were regenerated after interruption by hilar stripping, how transport of particles from the lung to LALN was changed by interruption and possible regeneration of lymphatics, and what the influence of this change was on antibody responses to particulate antigens in the lung.

Materials and methods

Experimental design

The experiments were divided into three parts: visual detection of regenerated lymphatics by chromolymphography, observation of transport of carbon particles from the lung to LALN and assessment of antibody responses after immunization in the hilar stripped lung.
Chromolymphography was performed on days 4, 7, 10 and 28 after hilar stripping (n = 2 on each day). Transport of carbon particles from the left lung to LALN was observed first in a control group of normal rats to determine the peak day on which carbon particles reached a maximum number in LALN. Therefore, the rats were sacrificed on day 1, 2, 3, 4, 5, 7 or 10 after instillation of carbon particles into left lungs (n = 4 on each day). Then, an experimental group of hilar stripped rats was instilled with carbon particles on the 4th, 7th, 10th, 14th or 28th postoperative day (n = 4 on each day). These rats were sacrificed on the peak day found in the control group. To assess antibody responses, rats were immunized with sheep red blood cells (SRBC) in normal left lungs and in hilar stripped lungs on day 7, 10, 14 or 28 after operation (n = 4 on each day). After immunization, serum antibody titers were followed during 1 month.

3.3 Pulmonary lymphdrainage in hilar stripped lungs

Rats

Male, pathogen-free Wistar rats weighing from 230 to 280 grams were obtained from Central Animal Facilities of Groningen University. All animals received humane care in compliance with the Dutch regulations and laws.

Hilar stripping

The technique of hilar stripping in rats has previously been described (4). In brief, after thoracotomy in the left fifth intercostal space, all of the surrounding tissues were dissected meticulously from the left main stem bronchus, pulmonary artery and vein, resulting in the complete disruption of the hilar lymphatics, the bronchial arteries and the branches of the vagal nerve to the left lung.

The rats were monitored after operation by chest roentgenography and body weight. Chest roentgenograms taken on days 4 and 7 postoperatively showed normal appearance of the lung. The weight of most rats decreased about 10 grams early after operation but returned to the preoperative values within 2 weeks.

Chromo-lymphography

Hilar lymphatics are so thin and transparent that they can hardly be seen. To make hilar lymphatics visible, we used chromolymphography. After thoracotomy, 0.05 ml of blue dye (Patent blue, Guernet) was injected directly into the lower left lung with a 25 G needle. Care was taken to avoid leakage of the dye into the pleural space. Hilar lymphatics became visible as the blue dye in the lung flowed through pulmonary lymphatics a few minutes after injection.

Intrabronchial instillation of carbon particles

To investigate transport of particles from the lung to LALN, carbon particles were selectively administered into left lungs by means of intrabronchial instillation. First, the rat was anesthetized by 4% halothane in a gas mixture of 30% oxygen and 70% nitrous oxide and intubated with a tracheal cannula (outer diameter 1.7 mm and inner diameter 1.25 mm), as described before (4). After that, a second curved catheter (outer diameter 1.0 mm and inner diameter 0.7 mm) with a metal-coated tip was introduced through the tracheal cannula

into the lower end of the left bronchus. The correct location of the tip of the intrabronchial catheter was checked by fluoroscopy. Then, 0.1 ml of India ink (Leitz) containing carbon particles was instilled via this intrabronchial catheter into the left lung. The intrabronchial catheter was removed immediately after instillation. The rat was placed on its left side, and the tracheal cannula was removed as soon as the rat woke up from anesthesia.

It was easy and uneventful to insert the tiny, curved catheter into the left bronchus and the introduction success rate checked by fluoroscopy was 100%. At sacrifice, all the left lungs without exception were stained with carbon particles, especially the lower part. Only occasionally right lungs were slightly stained.

Counting of carbon particles

At sacrifice, LALN as well as both lungs were examined macroscopically. All the lymph nodes in the left upper mediastinum and both lungs were then extirpated for histologic preparation. Sections were stained with nuclear fast red showing the carbon particles on a red background. Under the microscope, three areas were analysed in every lymph node, one randomly chosen in the medulla and two in the cortex on opposite sides of the lymph node. Macrophages containing carbon particles were counted using a microscopic counting grid. Data were expressed as number of the carbon particle-containing cells per counting unit.

Antibody response

Pilot studies showed that a dosage of 10^9 SRBC in 0.1 ml saline, instilled intrabronchially as carbon particles, evoked a clearly detectable antibody response in the peripheral blood. At intervals after immunization (4, 7, 10, 14, 21 and 28 days), blood samples were taken for antibody titer measurement. The total amount of anti-SRBC antibodies (IgM and IgG) in the peripheral blood was detected by a haemolysis assay. Titers were expressed as the ^{2}log of the last dilution showing haemolysis.

Statistical analysis

The numbers of transported carbon particles and detected antibody titers in normal and hilar stripped rats were compared with the Wilcoxon signed rank test. Differences were considered to be significant if the p-values were less than 0.05.

Results

Regeneration of lymphatics

In normal rats, hilar lymphatics were coloured blue and became readily visible after the injection of blue dye into left lungs. There were one or two hilar lymphatic trunks macroscopically, which connected to LALN in the left upper mediastinum, usually to the anterior lymph node located in front of the left superior vena cava. Rarely a lymphatic vessel was seen to connect with the posterior lymph node behind the vena cava.

On day 4 after hilar stripping, chromolymphography showed that the hilar lymphatics were interrupted. In one rat a regenerating lymphatic vessel was seen to extend 4 mm long beyond the hilum but without getting to a lymph node. On days 7 and 10 each, a small regenerated lymphatic vessel was recognizable and the anterior lymph node was faintly stained blue in one rat, but there were no lymphatics visible in the other rat. By day 28, regenerated lymphatics of normal size were seen distinctly in both rats. The path of the lymphatic vessel was the same as normal in one rat and changed toward the posterior lymph node in the other.

Transport of carbon particles

In normal rats, carbon particles appeared in anterior lymph nodes by 1 day and reached a maximum number between 4 and 7 days after instillation (Figure 1). Posterior lymph nodes contained no or small numbers of carbon particles. Based on this observation, in further experiments hilar stripped rats were sacrificed on day 4 after instillation.

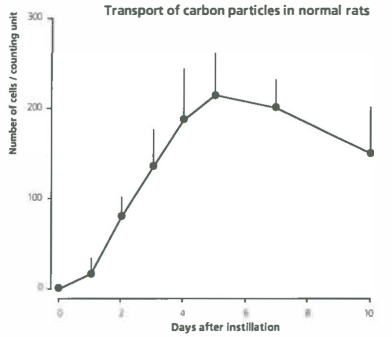

Figure 1. Transport of carbon particles from the lung to the lung-associated lymphnodes (LALN) in normal rats.

Carbon particles were intstilled into the left lung and rats were killed on different days after instillation (n = 4 on each day). Carbon particle-containing cells were counted microscopically in sections of the LALN. Data, expressed as number of cells per counting unit, are means±SD.

Carbon particles reached a maximum number in the LALN between 4 and 7 days after instillation.

In the hilar stripped rats instilled on the 4th postoperative day, carbon particles in LALN were markedly reduced in number compared with normal ($p < 0.05$). In the rats instilled on the 7th postoperative day, the number of carbon particles was low in two rats while approximately normal in the other two. In all the rats instilled on the 10th postoperative day, carbon particles were seen in almost normal quantities. By the 28th postoperative day, the number of carbon particles transported to LALN was completely normal (Figure 2). These numbers were reached mostly in anterior lymph node and twice in posterior lymph node.

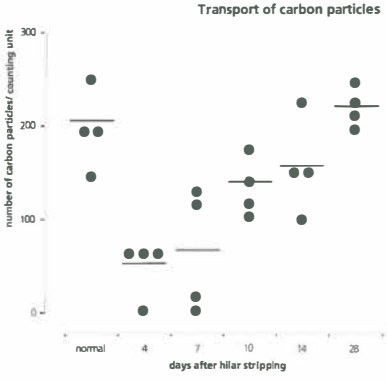

Figure 2. Transport of carbon particles from the lung to the lung-associated lymphnodes (LALN) in hilar-stripped rats.

Carbon particles were instilled into the left lung on different days after hilar stripping (n = 4 on each day) and rats were killed 4 days after instillation for counting of carbon particle-containing cells in the LALN (see legend of figure 1.). Each dot represents the value of an individuel rat. Horizontal lines are the average values.

The number of carbon particles in the LALN was lower than normal ($p<0.05$) in the rats instilled on day 4 after hilar stripping. On day 7, variation of the number of carbon particles was quite large between individual rats. By day 10, the number of particles in the LALN was almost normal.

3.3 Pulmonary lymphdrainage in hilar stripped lungs

Antibody responses

In normal rats, antibodies appeared in the blood on day 4 after immunization and reached a peak titer on day 10. After day 10 antibody titers were gradually falling (Figure 3).

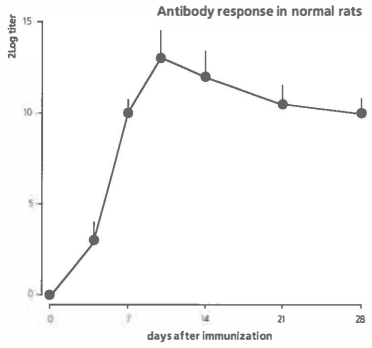

Figure 3. Antibody responses after lung immunization in normal rats (n = 4).

Rats were immunized with sheep red blood cells in the left lung and blood samples were taken on different days after immunization. Anti-SRBC antibody titers in the blood were detected by a hemolysis assay and expressed as the Log 2 of the last dilution that showed hemolyis. Data are given as means±SD. Serum antibodies reached a peak titer 10 days after immunization.

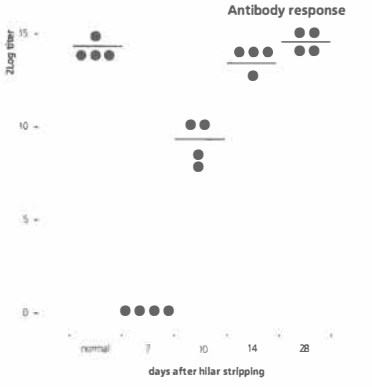

Figure 4. Antibody responses after lung immunization in hilar-stripped rats.

Rats were immunized with sheep red blood cells in the left lung on different days after hilar stripping (n = 4 on each day) and serum antibody titers were monitored during 1 month (see legend of Figure 3.). Each dot is the peak titer of an individual rat. Horizontal lines are the average values.

Peak titers were lower than normal in the rats immunized on day 7 ($p<0.01$) and on day 10 ($p<0.05$) after hilar stripping. Afterward, peak titers returned to normal values.

In hilar stripped rats, serum antibody responses were absent or weak when rats were immunized on day 7 after operation ($p < 0.01$). Responses became present but lower than normal ($p < 0.05$) when immunized on day 10 and completely normal by day 28 (Figure 4).

Two sham operated rats only underwent thoracotomy and handling of the lung. These two rats were immunized on day 7 after the procedure and showed normal antibody responses (peak titers were 12 and 13).

Discussion

The question whether the surgical procedure of lung transplantation might influence lung immune responses was confirmed in this study after hilar stripping in rats. Hilar stripping interrupted hilar lymphatics, it prevented transport of particles deposited in the lung, and it impaired antibody responses in LALN. All three aspects improved by 10 days and became normal one month after surgery.

Lymphatic regeneration has been previously studied following lung reimplantation in dogs by Eraslan and his colleagues (5). They injected sky blue dye into the reimplanted lung at various intervals following operation. They found that adjacent hilar lymph nodes were faintly stained at 7 days and a few small lymphatics were grossly visible by 12 days. These results are in concert with ours that hilar lymphatics were regenerated between 7 and 10 days after hilar stripping in rats. It indicates that our results obtained in hilar stripped rats are similar to those in transplanted large animals. Eraslan considered that the regeneration of lymphatics might play a significant role in dissipation of the pulmonary edema which usually follows lung reimplantation. In the present study, we found that pulmonary edema had vanished on chest films 4 days after hilar stripping, i.e. before lymphatic regeneration. Therefore, we stress that the regeneration of hilar lymphatics is essential for the transport of particulate antigens from the lung to LALN.

The changes in transport of carbon particles to LALN were consistent in time with the regeneration of hilar lymphatics. Carbon particles could not be transported during the first week after operation because of the interruption of lymphatics. Afterwards, the number of particles transported to LALN increased gradually as hilar lymphatics regenerated. Dal Col et al indicated in their experiment that surgical interruption of pulmonary lymphatics impaired the

translocation of microspheres instilled into the transplanted canine lung, but during a 14 postoperative day period they failed to observe that the translocation of microspheres recovered to normal levels (6). Our data demonstrated that newly regenerated lymphatics were able to transport carbon particles deposited in the lung to LALN in normal quantities one month after hilar stripping.

The antibody responses returned in parallel with the regeneration of lymphatics and the transport of particles. Our results showed that there was no antibody response as long as lymphatics were interrupted and the transport was blocked. This also confirms that antibody responses to antigens deposited in the lung are staged in LALN rather than inside the lung itself. Some overflow of SRBC from left to right could not be absolutely excluded, because carbon particles were seen occasionally on the right side. However , if this occurred, the number of SRBC in the right LALN was too low to induce a detectable antibody response, as in all the animals antibody responses were absent or weak early after hilar stripping.

In the sham operate rats, a normal antibody response could be induced after the procedure. This indicates that the surgical trauma itself did not reduce antibody responses after hilar stripping.

Conclusion

The interruptionof hilar lymphatics by hilar stripping severly impairs antibody responses in animal experiments. It is likely that lung transplantation will cause a similar impairment in patients. As a consequence of the reduced antibody responses, the lung transplant may be more susceptible to pulmonary infections. These findings may at least partly explain why pulmonary infections occur so frequently in the initial weeks after lung transplantation.

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3.4 Inadequate antibody response against respiratory viral infection in long-term surviving rat lung allografts

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Transplantation 1994, in press

J Heart Lung Transplant 1993; 12: S104 (abstract)

3.4 Inadequate antibody response against viral infection

Abstract

Lung transplant recipients suffer from a high number of viral infections. It has been suggested that the defense against viral infections is impaired in lung transplants. Therefore, we investigated in rat lung transplants whether antibody responses against an intrapulmonary viral infection were impaired in 3 groups of rats with: group 1: BN-to-LEW allogeneic lung transplants, group 2: LEW-to-LEW syngeneic lung transplants, and group 3: nontransplanted LEW lungs. All rats (including those with nontransplanted, normal, lungs) were treated with cyclosporine on days 2 and 3 after operation, which treatment is adequate to induce permanent graft acceptance of the allografts. Six months after transplantation viral infections with Sendai virus (parainfluenza type I) were induced intratracheally. At day 0, immediately prior to infection, and at days 4, 7, 21, and 56 after infection 4 rats in each group were killed for histological evaluation of the lungs. Number of antibody-positive cells in the bronchus associated lymphoid tissue (BALT) in the lungs and in the spleen, and presence of the virus in the lungs were determined by immunohistology. Serum antibody titers were followed for 56 days after infection.

The allogeneically transplanted lungs failed to respond adequately against the virus: the number of antibody-positive cells in the BALT did not increase after infection, serum antibody titers were hardly detectable; and virus was present in the airways of the lungs up to day 21 after infection. In contrast, in the syngeneically and nontransplanted lungs the number of antibody-forming cells in the BALT increased steeply till day 7, serum antibody titers rose till day 14; and virus could be detected only on day 4 after infection.

This study shows that in rat lung allografts both the local antibody production in the BALT and the systemic antibody response against a respiratory viral infection are inadequate. As a consequence the virus is longer present in these allografted lungs and can exert its damaging effect over a longer period of time. These results may explain why lung transplants are so susceptible to viral infections.

Introduction

Viral infections frequently affect the lungs of patients after heart-lung and lung transplantation and these infections are associated with high morbidity and mortality (1,2). Lung transplant recipients suffer from infections twice as often as heart transplant recipients despite comparable immunosuppressive protocols. Sofar, it is unclear why lung transplants are so susceptible to viral infections.

After a respiratory viral infection in the normal lung, antiviral antibodies are produced locally in the lung, in animals like rats and rabbits in the bronchus-associated lymphoid tissue (BALT), and systemically in the spleen (3,4). It is generally assumed that the local antibody production, especially the production of IgA in the BALT, is the most important first-defense against an intrapulmonary viral infection (3,4,5,6). Whether the antiviral antibody production is affected by transplantation of the lungs is not known. However, in long-term surviving rat lung allografts we found the cell density in the BALT to be decreased, possibly as a result of rejection episodes (7). Similarly, in a clinical study the number of immunoglobuline-positive cells in the submucosa of the transplanted lung was reduced in patients with chronic pulmonary rejection (8). This raises the question as whether in the transplanted lung a normal antibody response can be generated against viral infections.

In this study we therefore investigated the local and the systemic antibody responses and the clearance of virus from the airways after intrapulmonary infection with Sendai virus (parainfluenza type I) in long-term surviving rat lung transplants with chronic rejection (9).

Materials and methods

Experimental design

Antiviral antibody responses and presence of virus in the airways were investigated in long-term surviving lung transplants and in normal lungs after intrapulmonary infection with Sendai virus (parainfluenza type I). LEW rats were divided in three groups. In group 1 the rats received allogeneic BN lung transplants (n=20), in group 2 the rats

received syngeneic LEW lung transplants (n=20), and in group 3 the rats received no lung transplants (n=20). All rats were immunosuppressed with a cyclosporine (CsA) injection on days 2 and 3 after transplantation. The nontransplanted rats of group 3 received the same immunosuppressive treatment. Six months later 4 animals in each group were killed as non-infected controls (day 0). The remaining 16 rats of each group were infected intratracheally with Sendai virus. At day 4, 7, 21 and 56 after infection 4 infected animals in each group were killed for histological examination of the lungs and the spleens. Besides morphological evaluation of histopathology, the number of immunoglobuline-positive cells in the BALT and the spleen were determined semiquantitatively by immunohistology. Serum antibody titers were followed in the rats that survived till sacrifice at 56 days after infection. Clearance of the virus from the airways was determined by a monoclonal antibody staining against Sendai virus.

Rats

Young adult, male, specific pathogen-free LEW ($RT1^l$) and BN ($RT1^n$) rats, weighing 250-350 grams were obtained from Zentral-Institut für Versuchstiere, Hannover, Germany. All animals received humane care in compliance with the Dutch regulations and law.

Lung transplantation

Left lung grafts were orthotopically transplanted in the thorax, according to the improved technique of Marck and Prop (10). Briefly, the donor lung was dissected and its vascular bed flushed with cold saline. The recipient's left lung was removed and replaced with the donor lung; the pulmonary vein and artery were anastomosed first and than the bronchus.

To exclude technical failures, the transplanted lungs were monitored by chest roentgenography weekly during the first month and from then monthly untill the day of infection with Sendai virus. All chest roentgenograms showed normal appearence of the transplanted lung at the day of infection.

All rats received CsA (provided by Sandoz Pharmaceuticals Corporation, Basel, Switzerland), dissolved in olive oil, intramusculary in a dosage of 25 mg/kg body weight on days 2 and 3 after lung transplantation. This treatment is adequate to induce permanent graft acceptance of the lung allografts. Normal rats also received CsA for 2 days.

Virus

In this study Sendai virus (*Parainfluenza* type I) was used to induce a respiratory infection. Culture and preparation of Sendai virus were performed by the ICLAS Reference Centre for Rodent Viruses (Department of Microbiology, University Hospital Nijmegen, Nijmegen, The Netherlands) as previously described (11).

Sendai virus was injected intratracheally, at a dose of 10^3 plaque forming units in 0.2 ml medium. In a pilot study this virus-load induced mild pulmonary changes in normal LEW rats, with hyperplasia and lymphocytic infiltration of the bronchial epithelium and mild perivascular lymphocytic infiltration, which were transient.

Histology

For histological investigation of the lungs and spleens the rats were exsanguinated under ether anaesthesia. Heart and lungs were taken out from the thoracic cavity and the spleen was taken from the abdomen. The lungs were intratracheally infused with OCT (optimum cutting temperature) compound (Tissue-tek II: Lab-Tek Division. Miles Laboratories Inc. Naperville, IL) diluted 1:1 in phosphate buffered saline (PBS). Left and right lungs were separated at the hilar region and each lung was cut into two halves through the main bronchus to get longitudinal sections including the main bronchus. The spleen was cut in 2 mm-thick slices. One half lung and 3 slices of each spleen were embedded for paraffin sections. The other half lung and 3 slices of each spleen were snap frozen in liquid nitrogen and stored at - 80°C for immunohistological evaluation. Paraffin sections of lungs and spleens were stained for light microscopy with hematoxilin and eosin (H&E) and methyl green pyronine.

Immuno-histology

The antibody-positive cells in the BALT of the infected lungs and in the spleens were detected with the monoclonal antibodies MARM, MARG and MARA , directed against IgM, IgG and IgA containing cells, respectively (gift of dr. F. Kroese, Department of Histology and Cell Biology, University of Groningen, the Netherlands). Virus positive cells were stained with the monoclonal antibody MAB 834-3 directed against parainfluenza I virus (Chemicon International Inc, Temecula CA, USA). Therefore, serial cryostat sections were cut at 6 µm and air-dried for 30 min. The sections were rinsed in PBS and then incubated at room temperature for 1 hour with the appropriate monoclonal antibodies. After washing 3 times in PBS, sections were incubated for 30 minutes with horseradish-peroxidase-conjugated rabbit-antimouse immunoglobulin (DAKO, Denmark). Peroxidase was revealed by staining with 3,3'-diaminobenzidine tetrachloride. Sections were lightly counterstained with hematoxylin. To assess nonspecific staining, control sections were incubated with PBS instead of monoclonal antibodies.

Assessment of cell numbers

The number of antibody-positive (IgA, IgG and IgM) cells in sections of the BALT and spleen was counted using a grid micrometer with 100 squares at a magnification of x400. The number of antibody forming cells was counted in 6 randomly selected areas in each BALT and in 6 B cell areas in each spleen of each rat and their numbers were added. Giving a total number for each rat. This number was used for statistical analysis.

The number of virus positive cells were scored in a semiquantitative way in four randomly selected areas of bronchial and bronchiolar epithelium in each lung, recognising 4 cell scores in which - = no cells, + = 1 to 10 positive cells, ++ = 10 to 20 positive cells and +++ = more than 20 positive cells per field of view at a magnification of x200. For each lung a median score was calculated.

Serum antibody titer

Immediately prior to infection (day 0) and at intervals after infection (4, 7, 14, 21, 28, 42 and 56 days) blood samples for antibody titer measurements were obtained by retro-orbital puncture. The titer of Sendai-specific antibodies was determined with an ELISA titer plate assay using the direct binding method. Alternate columns of the titer plate were coated with parainfluenza virus or bovine albumine as control. Serum was added at a 1:50 dilution and serially diluted. Then enzyme-labeled goat anti-rat Ig was used as detecting agent. Titers were calculated based on the 2log transformation of the last dilution showing positive reaction.

Statistical analysis

Means and standard deviations of the number of immunoglobuline-positive cells in the BALT and in the spleen were calculated for the different groups on the different time-points. Mean systemic antibody titers and standard deviations in the different groups were calculated on the basis of the 2log titers of the individual titers. Number of antibody forming cells, serum antibody titers and the number of virus positive cells in the lungs in the different experimental groups were compared with the Mann-Whitney rank sum test for unpaired values and the number of antibody forming cells and the number of virus positive cells in the left and right lungs with the Wilcoxon signed rank test for paired values. A p value of less than 0.05 was considered statistically significant. All statistical calculations were performed with the statistical software package StatviewII™ for the Apple Macintosh computer.

Results

Local antibody response in the BALT

Morphology

In the non-infected rats (day 0), the BALT of the allogeneically transplanted lungs was cell-poor and small compared with the BALT in the syngeneically transplanted and nontransplanted lungs. After infection no morphological immune response developed in the BALT of the allogeneically transplanted lungs. Activated lymphocytes and plasmacells did not appear in the BALT during the observation period of 56

days after infection (fig 1A). As a consequence the BALT in these allogeneically transplanted lungs did not increase in size. In the BALT of the syngeneically transplanted and normal lungs a massive morphological response started on day 4 after infection with the appearance of lymphoblasts. At day 7 after infection large numbers of pyroninophylic lymphoblasts were present throughout the BALT. From day 21 through day 56 after infection, plasma cells were present in high numbers in the BALT. During the strong morphological immune response the size of the BALT increased considerably and remained enlarged up till day 56 after infection (fig 1B).

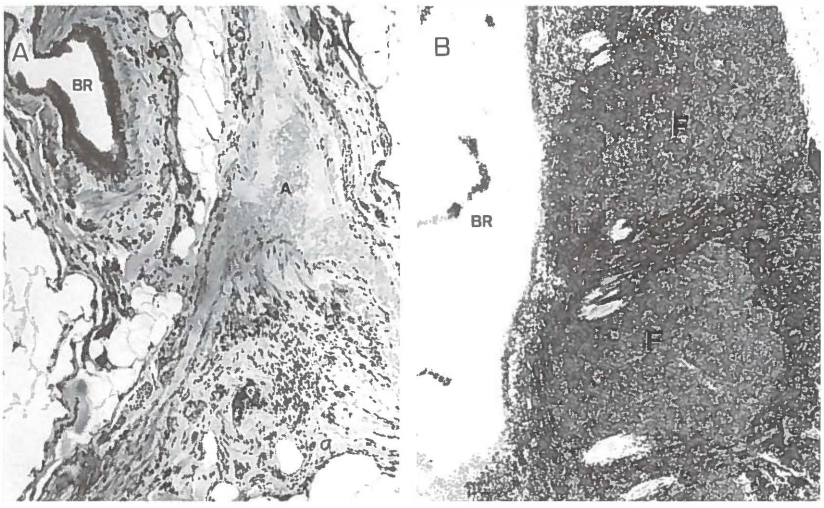


Figure 1. Morphological immune response in the BALT after respiratory viral infection in allogeneic lung transplants (A) and syngeneic lung transplants (B) on day 56 after infection.
(H&E, original magnification x60).

A Allogeneic lung transplants. After infection no immune response developed in the remnants of the BALT of these lungs (BR is bronchus, A is bronchial artery). Activated lymphocytes and plasmacells did not appear in the BALT. As a consequence, the BALT did not increase in size.

B. Syngeneic lung transplants. In the BALT of these lungs a massive immune response developed, with the appearance of follicles (F), containing lymphocytes in different stages of activation and plasmacells (BR is bronchus). During this strong immune response the size of the BALT increased considerably.

Cell numbers Assessment of antibody-positive cells showed that already before infection the total number of antibody-positive cells in the BALT of the allogeneicaly transplanted lung was significantly lower ($p<0.05$) than in the syngeniecally transplanted and nontransplanted lungs (fig 2).

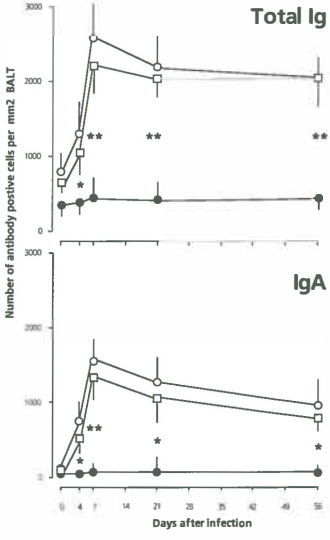
After infection the number of cells in the BALT positive for total Ig or IgA did not increase in allogeneically transplanted lungs (fig 2). In the syngeneically transplanted and nontransplanted lungs the number of Ig posititve cells in the BALT started to increase already on day 4 after infection. In particular, the number of IgA positive cells increased quickly in the syngeneically transplanted and nontransplanted lungs (fig 2). These numbers remained high up to 56 days after infection. The results of the syngeneically transplanted lungs were in striking parallel with the results of the nontransplanted lungs.

The contralateral right (nontransplanted) lungs in all groups showed an increase in antibody-positive cells in the BALT which was the same as that in the nontransplanted left lungs.

Figure 2. Number of antibody-positive cells in the BALT of the lung after viral repiratory infection.

After infection the number of cells in the BALT positive for total IG or IgA did not increase in the allogeneically transplanted lungs (closed circles). In hte syngeneically transplanted lungs (open circles) and nontransplantd lungs (open squares) the number of Ig-positive cells in the BALT started to increase already on day 4 after infection. In particular, the number of IGA-positive cells increased quickly in the BALT of these lungs. Ig and IgA cell numbers were significantly lower in the allogeneically transplanted lungs than in the syngeneically transplanted lungs on all corresponding time-points.

(* = $p<0.05$, ** = $p<0.01$).



Systemic antibody response

Morphology Light microscopy of the spleens in all three groups showed a typical immunoproliferative response in the B cell areas (germinal centers and corona) and in the T cell areas (PALS). In the allogeneically transplanted rats the intensity of the systemic immune response was weaker than in the syngeneically transplanted and nontransplanted rats: in the allogeneically transplanted rats the plasma cell reaction was weak and the T cell response involved only parts of the PALSes. This resulted in smaller germinal centers in the spleens of the allogeneically transplanted rats than in the other groups.

Cell numbers The number of antibody-positive cells in the spleens of the allogeneically transplanted group did increase, but was lower than in the syngeneically transplanted and normal groups ($p<0.05$ on days 7 and 21) (fig 3), which corresponds with the

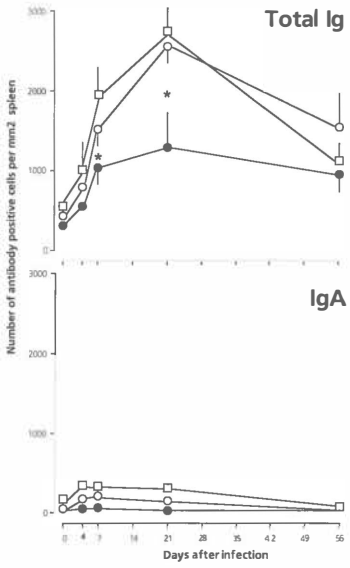


Figure 3. Number of antibody-positive cells in the spleen after viral repiratory infection.

The total number of antibody-positive cells in the spleens of the allogeneically transplanted group (closed circles) did increase, but the increase was lower than in the syngeneically transplanted (open circles) and nontransplanted (open squares) groups (* = $p<0.05$ on days 7 and 21). The number of IgA-positive cells did virtually not increase in either of the three groups, without any difference between the groups.

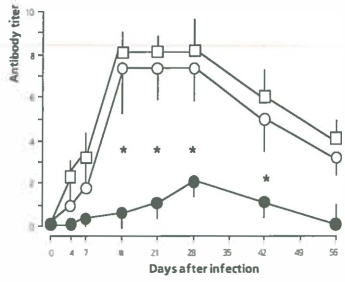
weaker germinal center reaction in the allogeneically transplanted group after viral infection. In contrast to the BALT, the number of IgA forming cells in the spleen remained low in all groups.

Serum antibody titer

Serum antibody titers after infection in all groups corresponded to the number of antibody forming cells in the spleen. The antibody titers were significantly lower in the allogeneically transplanted group than in the syngeneically transplanted and nontransplanted group ($p<0.01$ on from day 7 after infection) (fig 4).

Figure 4. Serum antibody titers after respiratory viral infection detected with ELISA assy.

The serum antibody titers were significantly lower in the allogeneically transplanted group (closed circles) than in the syngeneically transplanted (open circles) and nontransplanted (open squares) groups ($p<0.01$ from day 7 after infection).



Clearance of virus positive cells from the bronchial epithelium

Virus-positive cells could clearly be detected in the bronchial epithelium on day 4 after infection in all infected lungs (fig 5A and 5C). The distribution of the virus was more widespread in the allogeneically transplanted lungs than in the syngeneically and nontransplanted lungs, affecting also the bronchioles (table 1). Furthermore, clearance of the virus from the epithelium was slower in the allogeneically transplanted lungs than in the syngeneically transplanted and nontransplanted lungs (table 1).

In the allogeneically transplanted lungs virus-positive cells were still present in the epithelium of the airways on day 21 after infection (fig 5B). In contrast, in the syngeneically transplanted and normal lungs virus positive cells could not be detected in the epithelium of the airways anymore after day 4 after infection (fig 5D).

Table 1. Presence of virus positive cells in the airway epithelium

Days after infections	0	4	7	21	56
Allogeneically transplanted rats					
Left lungs					
Large airways	-	++	++	+	-*
Bronchioles	-	++	++	+/-	-*
Right lungs					
Large airways	-	+	-	-	-
Bronchioles	-	+/-	-	-	-
Syngeneically transplanted and nontransplanted rats					
Left lungs					
Large airways	-	+	-	-	-
Bronchioles	-	+/-	-	-	-
Right lungs					
Large airways	-	+	-	-	-
Bronchioles	-	+/-	-	-	-

For scoring see materials and methods. Virus positive cells were significantly longer present in the epithelium of the allogeneically transplanted lungs than in the airways of the syngeneically transplanted and nontransplanted lungs (* = $p<0.05$ with Mann-Whitney Rank Sum Test)

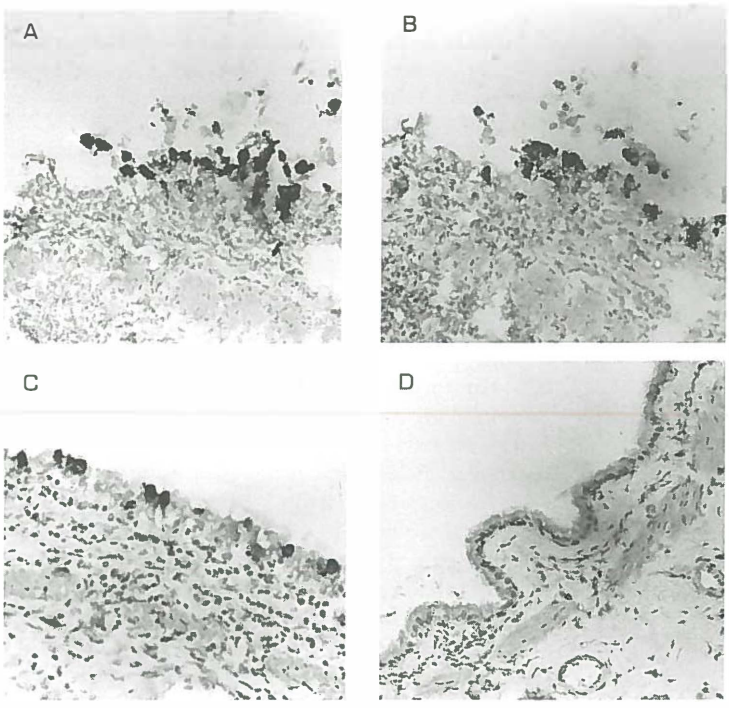


Fig 5A-D. Presence of virus in the bronchial epithelium of allogeneic lung transplants (A and B) and syngeneic lung transplants (C and D), 4 days after infection with Sendai virus (A and C) and 21 days after infection (B and D).
(Cryostat sections stained with a monoclonal antibody against Sendai virus, original magnification x 160).

Allogeneic lung transplants. A. Day 4 after infection. Large numbers of dark virus containing cells could be detected in the large airways. The epithelium is hyperplastic and necrotic. **B.** Day 21 after infection. Significant numbers of virus containing cells could still be detected in the large airways. The epithelium is still hyperplastic and disrupted.

Syngeneic lung transplants. C. Day 4 after infection. Virus containing cells could be detected clearlyin the epithelium of the large airways, but in lower number than in the allogeneic lung transplants. The epithelium is hyperplastic, but not necrotic. **D.** Day 21 after infection. Virus could not be detected in the epithelium anymore. The epithelium is completely normal.

The contralateral right lungs in all groups showed a similar pattern of presence of the virus as the syngeneically transplanted and normal left lung: also in these lungs virus was only found on day 4 after infection.

Discussion

This study demonstrates that in rats with allogeneic lung transplants both the local antibody response in the BALT and the systemic antibody response against a respiratory viral infection are inadequate. This is supported by the finding that the virus is cleared more slowly from the lung allografts than from syngeneically transplanted lungs and nontransplanted lungs.

In normal lungs, an important component of the defense system against respiratory viral infections has been attributed to antibody production locally in the BALT (3,4,5,12). Especially local IgA production is important in preventing infection by the virus of the bronchial epithelium by blocking of viral antigens (3,4,5). It has been shown that patients with an impaired IgA production are predisposed to pulmonary viral infections (5,13). Our present study demonstrated that allogeneic rat lung transplants are not capable to generate an antibody response in the BALT. After infection with Sendai virus in the allogeneic lung transplants a morphological immune response in the BALT did not occur and the number of IgA-containing cells in the BALT did not increase. A similar observation has been made in a study on the immune responses in rat small bowel transplants (14). In that study no local IgA antibody response occured in the Peyer's patches (the local lymphoid tissue in the small bowel) after immunization with cholera toxin. Taken together, these results suggest that the local lymphoid tissue, ie BALT in the lungs, does not function properly after allogeneic transplantation.

Explanations for the impaired function of BALT in lung allografts may be found in the structural changes of its lymphoid tissue. Already prior to infection the surface area and cell density of the BALT (7), and the number of

immunoglobulin containing cells in the BALT (fig 2) are decreased in the allografts. Two causes have been suggested to be responsible for these structural changes, considering the fact that these changes do only occur in the allogeneic and not in the syngeneic lung transplants. First, the BALT in the lung allografts may be damaged by rejection because of its high immunogenecity (15). Although BALT is not a constituent structure in human lungs, two clinical studies suggested that also after human lung transplantation lymphoid tissue in the transplanted lung is damaged. In one report a decrease in the number of immunoglobulin containing cells in the submucosa of transplanted lungs was associated with chronic rejection (8). Another clinical study reported fibrosis of concomittantly transplanted hilar lymphnodes in heart-lung transplants with rejection episodes (16). Second, the BALT may be depleted of lymphocytes if migration of recipient lymphocytes to the BALT in the lung allografts is disturbed. To our knowledge no studies have been published about migration of recipient lymphocytes into the BALT of allogeneic lung transplants. Results from a current animal study indicate that fibrosis of the BALT in long-term surviving allogeneic rat lung transplants hampers normal migration of recipient lymphocytes indeed (17). It is quite conceivable that these structural changes prevent a proper function of BALT after respiratory viral infections.

Besides the local antibody response, also the systemic antibody response in spleen and blood was impaired in the allografted animals; the cause of it is unclear. One could imagine that already the induction of antiviral responses in the spleen is affected in allografted rats. As mentioned above, the numbers of recipient lymphocytes migrating through the lung allograft, or at least through its BALT, are largely reduced. On top of that, the uptake of antigens from the airways into the BALT has been found to be blocked, probably by a fibrotic layer isolating the BALT (17). In this way a proper surveillance and peripheral sensitisation of lymphocytes may be prevented. Absence of peripheral sensitisation will prevent

the induction of the systemic response against respiratory infections. One can also imagine that the systemic response is affected after its induction by a suppressive bystander effect: suppressor mechanisms of the alloresponses, involved in maintaining the graft, interfere with the anti-viral responses. Bystander suppression of alloresponses and other immune responses have been shown in various experimental models (18). In parallel, we found an equal systemic suppression of antiviral responses when rats with long-term surviving heart and spleen allografts (19) were intrapulmonary infected with Sendai virus: the systemic antibody response was equally low as in the allografted group in this study (unpublished observations). Irrespective of the cause of the reduced systemic antibody reponse, it seems to have little influence on the clearance of the Sendai virus from the airways. This is demonstrated by our present finding that virus was cleared normally from the contra-lateral, right nontransplanted lung in the allogeneically transplanted animals. We attribute the normal clearance of the virus to the adequate antibody production in the BALT of these contra-lateral right lungs. These findings are an illustration of the idea that a proper function of the BALT is most essential for an adequate response against a respiratory viral infection.

Not all responses against intrapulmonary antigens are suppressed in lung allografts. In a previous study we found that a normal serum antibody titer was generated after instillation of sheep red blood cells in long-term surviving rat lung allografts (20). These antibodies, however, are not produced in the BALT of the donor lung, but in the paratracheal lymphnodes (PTL) (21). Also the induction of this response in the paratracheal lymphnodes is independent of the BALT, because these antigens are transported directly through lymph vessels to the lymphnodes (21). Whether also T cell responses are normal in lung allografts cannot be excluded because this was not investigated specifically in the present study. However, it is unlikely that they are normal because morphological T cell responses were absent in the

BALT and largely reduced in T cell areas of the spleen. T cell responses are more important for defense against viral infections such as CMV (22) than against respiratory viral infections which heavily depend on IgA antibodies for primary defense.

A consequence of the impaired antibody responses against respiratory viral infections in the allogeneic lung transplants is that the virus is present in the airways over a prolonged period of time. During its prolonged presence in the airways, the virus can initiate an excessive inflammatory reaction with subsequent severe airway damage. In a previous study this airway damage after viral infection in lung allogeneic lung transplants appeared to be very severe, indeed (23). We think that this severely damaging effect of viral infections in allogeneic lung transplants may contribute to the development of obliterative bronchiolitis, which is the major complication after clinical lung transplantation (24).

From this study it is clear that the BALT in allogeneically transplanted lungs is not capable to generate an adequate antibody response against respiratory viral infections. As a consequence the virus is longer present in these allografted lungs and can exert its damaging effect over a longer period of time. These results may explain why lung transplants are so susceptible to viral infections and why viral infections cause severe damage in these lung transplants.

3.4 Inadequate antibody response against viral infection

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3.5 Defective bronchus-associated lymphoid tissue in long-term surviving rat lung allografts

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3.5 Defective BALT in lung allografts

Abstract

In a previous study we found that the defense against respiratory viral infections is inadequate after allotransplantation of the lung: no local immune response and subsequent production of antibodies developed in the bronchus-associated lymphoid tissue (BALT) of infected rat lung allografts. We hypothesised that the BALT in long-term surviving rat lung allografts was unable to function adequately because it was somehow damaged by the transplantation. In the present study, we investigated 3 prerequisites for a normal function of the BALT, i.e. its structure, the uptake of antigens and the lymphocyte migration to the BALT. These 3 prerequisites were investigated in 3 groups of rats (n=10 each): group 1: BN-to-LEW allografts; group 2: LEW-to-LEW isografts and group 3: normal LEW rats. All rats were immunosuppressed with CsA (injected on days 2 and 3). Six months after transplantation the BALT was investigated. The structure of the BALT and the uptake of intrabronchially injected carbon particles in the BALT were determined histologically. The migration of intravenously injected, FITC-labelled thoracic duct lymphocytes to the BALT were determined immunohistochemically.

In the allografts the BALT was defective in all 3 investigated aspects. It was reduced in size and lymphocyte density and was largely replaced by fibrous tissue. The fibrosis hampered uptake of particles from the airways and lymphocyte migration to the BALT: 24 hours after administration no carbon particles and only few labelled lymphocytes were found in the BALT. In contrast, in the syngeneically transplanted and nontransplanted lungs the BALT consisted of a large and dense collection of lymphocytes closely associated with the bronchial epithelium. In these BALTs large numbers of carbon particles and labelled lymphocytes were found.

In conclusion, after allogeneic transplantation the BALT in the lung becomes defective in respect of structure and function. This is most likely caused by rejection, as the BALT in syngeneic lung transplants was perfectly normal. Hence, we speculate that the local immune response may be preserved by better immunosuppressive regimens.

Introduction

Despite increasing experience in the management of lung transplant recipients, pulmonary infections continue to be major complications early and late after (heart-) lung transplantation (1,2). In a previous study (3) we concluded that the defense against intrapulmonary infections is inadequate after allogeneic transplantation of the lung. In that study we found that no local immune response develops after viral parainfluenza infection in allogeneically transplanted rat

lungs: the bronchus-associated tissue (BALT) of these allografts did not produce antibodies. As a consequence the virus was present for a longer time in the allografted lungs than in normal lungs.These findings suggested that the BALT in lung transplants was unable to react adequately against intrapulmonary antigens. We postulated that it has become defective after allotransplantation as a result of localised rejection.

In the normal lung the BALT forms a local immune system in a number of mammalian species, including man (4,5). It is predominantly localised along the main bronchi, between the bronchus and its accompanying pulmonary artery. The BALT consists of a collection of lymphoid cells closely associated with the bronchial epithelium and submucosal tissue. The main functions of the BALT are antigen-uptake from the airways and the local production of IgA, IgM and IgG (4,5,6). After antigenic stimulation in the BALT more lymphocytes are recruited from the circulation and involved in the immune response in the BALT. Since the BALT has no afferent lymph vessels lymphocytes can only reach the BALT via blood vessels, in particular through high endothelial venules (HEVs) (7), which are entry sites for lymphocytes in many other lymphoid organs, too (8).

After lung transplantation the BALT has been shown to be the primary site of acute rejection (9). Even when acute rejection episodes are adequately treated, the structure of the BALT seems to be affected by localised (chronic) rejection as we observed in long-term surviving rat lung allografts (10). It is conceivable that damage of the structure of the BALT after lung transplantation does disturb antigen uptake in the BALT or the lymphocyte migration to the BALT. Either disturbance, or a combination of both might explain how the generation of an antibody response in the BALT, e.g. after a viral infection (3), is hampered. Therefore we investigated in this study whether the structural components of the BALT, uptake of particles in the BALT and the migration of lymphocytes to the BALT are changed after lung transplantation in the rat.

In order to avoid influence of active acute rejection on the experiments, the BALT was investigated in long-term surviving lungs six months after transplantation. The BALT was investigated in allogeneically transplanted lungs, in syngeneically transplanted lungs and in nontransplanted lungs.

Materials and Methods

Experimental design

The experiments were divided into three parts:
A: histological evaluation of the structure of the BALT,
B: uptake of carbon particles from the lung in the BALT and
C: assessment of migration of lymphocytes to the BALT. The experiments were performed in three groups of LEW rats (n=10 each). In the first group the rats received BN allogeneic lung transplants, in the second group the rats received LEW syngeneic lung transplants and in the third group the rats received no lung transplants. All rats were immunosuppressed with Cyclosporine-A (CsA), given on days 2 and 3 after transplantation. The normal rats of the third group received the same CsA treatment. Six months after transplantation and CsA treatment the BALT was investigated.

In each group the structure of the BALT was investigated in 7 rats, the uptake of carbon particles in the BALT was determined in the remaining 3 rats and the migration of fluorescein isothiocyanate (FITC)-labeled lymphocytes to the BALT was determined in the same rats that were used to investigate the structure of the BALT.

Rats

Young adult, male, specific pathogen-free LEW ($RT1^l$) and BN ($RT1^n$) rats, weighing 250-350 grams were obtained from Zentral-Institut für Versuchstiere, Hannover, Germany. All animals received humane care in compliance with the Dutch regulations and law.

Lung transplantation

Left lung grafts were orthotopically transplanted in the thorax, according to the improved technique of Prop and Marck (11). Briefly, the donor lung was dissected and its vascular bed flushed with cold saline. The recipient's left lung

was removed and replaced with the donor lung; the pulmonary vein and artery were anastomosed first and than the bronchus.

To exclude technical failures, the aeration of transplanted lungs was monitored by chest roentgenography weekly at the first month and from then monthly untill use in the experiments. All chest roentgenograms showed a normal appearence of the transplanted lung at the day of the experiments.

All rats received CsA (provided by Sandoz Pharmaceuticals Corporation, Basel, Switzerland), dissolved in olive oil, intramusculary in a dosage of 25 mg/kg body weight on days 2 and 3 after lung transplantation. This treatment is known to be adequate to induce permanent graft acceptance of the allogeneic lung transplants (12). The rats of the nontransplanted group received the same treatment.

Lung tissue

For histological and immunohistological investigation of the lungs rats were exsanguinated under ether anaesthesia. Heart and lungs were taken out en bloc from the thoracic cage. The lungs were intracheally infused with a 50% solution of OCT (optimum cutting temperature) compound (Tissue-tek II: Lab-Tek Division. Miles Laboratories Inc. Naperville, IL) in phosphate buffered saline (PBS). Both lungs were separated at the hilar region and cut into two halves through the main bronchus to get longitudinal sections including the bronchus. In this technique BALT is always present in sections of the lung. One half of the lung was embedded for paraffin sections and the other half lung was snap frozen in liquid nitrogen and stored at - 80 C. Paraffin sections were cut at 6 µm and stained for light microscopy with hematoxyllin and eosin (H&E).

For immunohistological staining serial cryostat sections were cut at 6 µm and air-dried for 30 min. The sections were rinsed in PBS and then incubated at room temperature for 1 hour with the appropriate antibodies (see below). After washing three times in PBS, sections were incubated for 30 min with horseradish-peroxidase-conjugated rabbit anti-mouse immunoglobulin (Dakopatts, Denmark). Peroxidase was

revealed by staining with 3,3'-diaminobenzidine-tetrachloride (Sigma) dissolved in PBS at a concentration of 1mg/ml containing 0.02% H_2O_2 . The sections were counterstained with hematoxillin, dehydrated and mounted. To assess nonspecific staining, control sections were incubated with PBS instead of monoclonal antibodies.

Immuno-histology

Two monoclonals were used in this study. The monoclonal antibody HIS22 was used to detect high endothelial venules (HEV) and anti-FITC was used to detect FITC-labeled lymphocytes. Both monoclonal antibodies were a gift from Dr. F. Kroese Dept. of Histology and Cell Biology, University of Groningen, The Netherlands.

Histology

The structure of the BALT was evaluated histologically in H&E sections of all lungs. In all lungs BALT could clearly be detected between the main bronchus and a pulmonary artery (fig 1A and 1B). Furthermore, in the lung sections the BALT's surface area, cell density and the number of high endothelial venules were determined quantitatively.

The surface area of the BALT was determined using a grid type micrometer (100 squares) at a magnification of x 100. At this magnification the grid covers 1 mm^2. The total surface area of the BALT per lung section was scored as the number of squares fully covering BALT and is expressed in mm^2.

The cell density of the BALT was assessed with the grid type micrometer at a magnifaction of x 400. In each square the number of lymphocytes was counted. A total of 600 squares was counted per lung. The cell density of the BALT is presented in a histogram with distribution intervals of 5 cells per square of BALT.

The number of high endothelial venules in the total BALT area in a section was counted at a magnification of x100; numbers of HEV in BALT are given as total number per lung section.

Uptake of carbon particles

To investigate the uptake of particulate antigens from the airways into the BALT, carbon particles were administered directly into the left lungs of 3 rats in each group by means of an intrabronchial catheter as we described before (12). This was done to ensure identical amounts of particles in each lung by excluding the interference of a possible bronchial stenosis after lung transplantation. After correct location of the tip of the catheter in the bronchus of the graft, guided by fluoroscopy, 0.1 ml of carbon particles containing Indian ink (E. Leitz KG, Hamburg, Germany) was instilled into the left lung. Twentyfour hours later the rats were sacrificed for histologic examination of the lungs. Lung sections were stained with nuclear fast red biologic stain, showing the carbon particles on a red background. The total number of carbon particles taken up into the BALT was counted in the whole BALT area of the left lung section and expressed as the number of carbon particles per mm^2 BALT.

Migration of Lymphocytes to the BALT

Migration of lymphocytes to the BALT was determined by intravenous injection of FITC-labeled lymphocytes and analysing the number of immigrated cells in the BALT 24 hours later. At this time-point migration and recirculation of lymphocytes is stabilized (13).

Lymphocytes used for migration were obtained by thoracic duct cannulation in normal LEW rats (mean body weight 300 ± 50 g). The thoracic duct was cannulated according to the method of Ford and Hunt (14). The lymph was collected overnight at 4 C into continuously shaken glass bottles which contained 5-10 ml Dulbecco's A+B (DAB) medium (Oxoid, Basingstoke, England) and 20 U heparin/ml medium . During the lymph sampling period the rats received intravenous infusions of 3 ml/h DAB with 1 U heparin/ml medium. After spinning down of the lymphocytes, contaminating red cells were lysed by osmotic shock. The cells were suspended at $50x10^6$/ml in RPMI 1640 and incubated for 15 min at 37 C with (FITC, Isomer 1, Sigma) at a final concentration of 50 µg FITC/ml and 2% fetal calf serum (FCS). The cells were centrifuged through a FCS cushion, suspended in PBS, and

counted. The viability always exceeded 95% measured by eosin Y exclusion. A total of 100 x10^6 cells per rat were injected through a venous cannula inserted into the tail vein. Twentyfour hours after injection, left and right lungs were taken for histological and immunohistological evaluation as described above. The number of immigrating FITC-labeled lymphocytes were counted in 2 ways. First, the 'absolute' number of FITC-labeled lymphocytes of a section was counted in the total BALT at a magnification of x100 with a grid micrometer. The 'absolute number' of immigrating lymphocytes was expressed as the number of FITC-labeled lymphocytes per mm^2 BALT. Second, the percentage of FITC-labeled lymphocytes of the total number of lymphocytes was determined in selected areas of the BALT. In the allografted lungs we selected those remnants of the BALT having the highest cell density. In the syngeneically transplanted and nontransplanted lungs these areas were randomly selected. In these areas of the BALT the number of FITC-labeled lymphocytes and the total number of cells were counted at a magnification of x400. The 'relative' number of FITC-labeled lymphocytes was expressed as the percentage of the total number of lymphocytes in these areas.

Statistical analysis

Differences between the different groups were compared with the Mann-Whitney rank sum test for unpaired values and between the left and right lungs with the Wilcoxon signed rank test for paired values. A p value < 0.05 was regarded statistically significant.

Results

Structure of BALT

The structure of the BALT in the allogeneic lung transplants (fig 1A) was clearly different from the syngeneically transplanted and nontransplanted lungs (fig 1B). In the allogeneic lung transplants the BALT was less prominent and more difficult tot recognize than in syngeneically transplanted lungs. It consisted of areas of lymphoyctes loosely dispersed in fibrous tissue, in contrast to the dense accumulation of

lymphoid cells in the BALT of the syngeneically transplanted and non-transpanted lungs. In the BALT of all allogeneic lung transplants a fibrous layer in the subepithelial region separated the lymphoid cells from the overlying epithelium; the muscularis mucosae had disappeared. This fibrous layer was not present in the BALT of the syngeneically transplanted and nontransplanted lungs, where BALT was situated directly under the bronchus epithelium; the muscularis mucosae was present in the BALT of these lungs.

The BALT of the contralateral right lungs in the allografted rats had a similar appearance as BALT in the syngeneically transplanted and nontransplanted lungs.

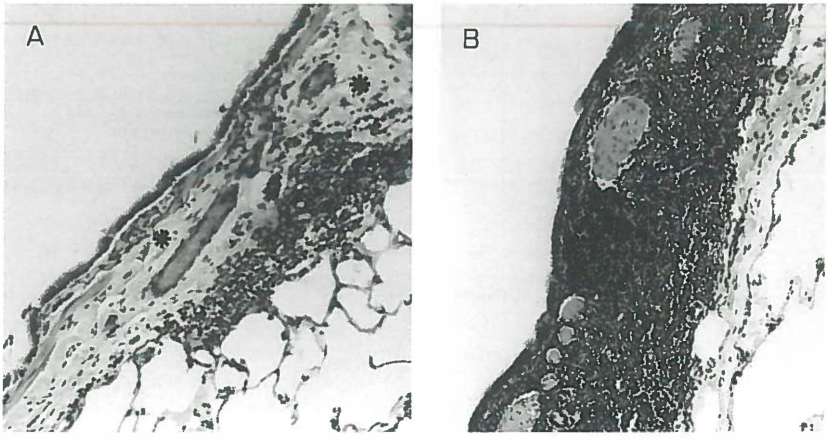


Figure 1. Structure of the BALT in allogeneic lung transplants (A) and syngeneic lung transplants (B). (H&E, original magnification x 60).

A. Allogeneic lung transplants. Small numbers of lymphocytes are dispersed in fibrous tissue. A fibrous layer (asterisks) separates the lymphoid cells from the overlying epithelium.

B. Syngeneic lung transplants. A dense collection of lymphoid cells is situated directly under the bronchial epithelium. Lymphocytes infiltrate into the lymphoepithelium.

3.5 Defective BALT in lung allografts

Surface area The surfacae area of the BALT was significantly smaller ($p<0.01$) in the allogeneic lung transplants than in the syngeneically transplanted and nontransplanted lungs (fig 2).The BALT of the syngeneically transplanted lungs had a similar surface area as the BALT of the nontransplanted lungs. Also, the surface area of the BALT in the contralateral right nontransplanted lung in the allografted rats was unchanged.

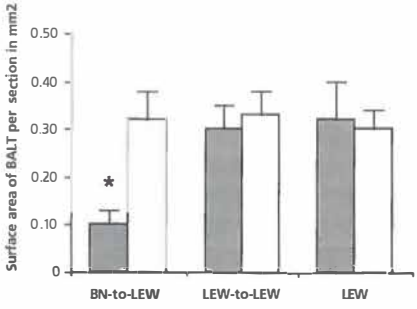


Figure 2. Surface area of the BALT.

The surface area of the BALT in the allogeneic (BN-to-LEW) left lung transplants is significantly smaller (* = $p<0.01$) than in the syngeneically transplanted (LEW-to-LEW) and nontransplanted (LEW) lungs. The surface of the BALT in the contralateral right, nontransplanted lung in the allografted rats is unchanged.

Left lungs: dark gray shaded columns, Right lungs: light gray shaded columns

Cell density The cell density of the BALT was lower in the allogeneic lung transplants than in the syngeneically transplanted and in the nontransplanted lungs (fig 3). The histogram of the cell density of the BALT in the allogeneically transplanted lungs showed a shift to the left and lacked columns representing areas of high cell density. The percentage of squares containing 0 to 4 and 5 to 9 lymphocytes was signficantly higher ($p<0.05$) in the allogeneically transplanted lungs than in the syngeneically transplanted and nontransplanted lungs. Only small areas with a normal cell density were present in the BALT of the allogeneic lung transplants. No such shift in cell density occured after syngeneic lung transplantation: the BALT in these lungs had the same density as the BALT in the nontransplanted lungs. In the contralateral right nontransplanted lungs of all three groups the cell density of the BALT was the same.

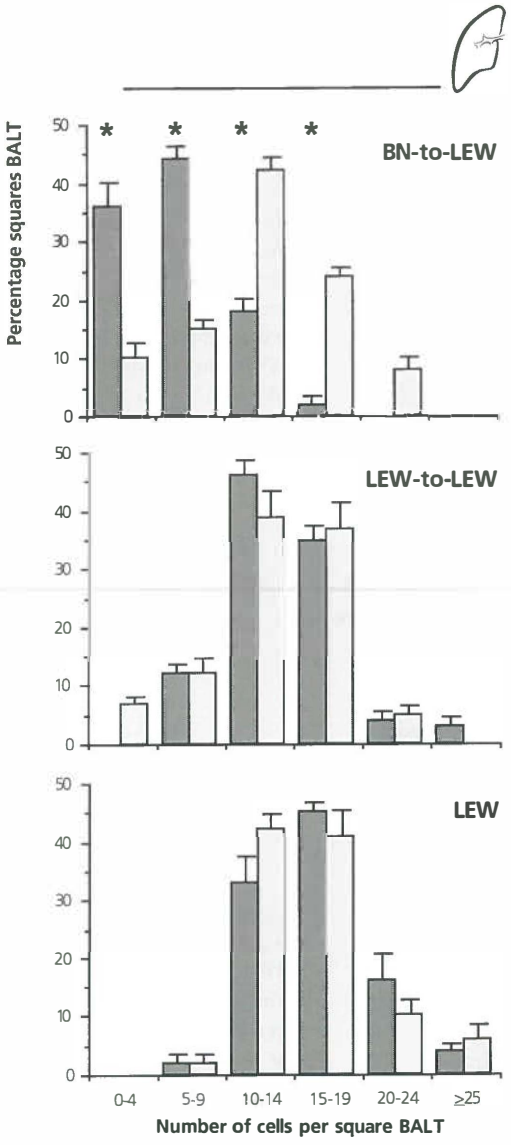


Figure 3. Cell density of the BALT

The cell density of the BALT is represented in histograms with distribution intervals of 5 cells per square BALT. The cell density of the BALT is lower in the allogeneic lung transplants than in the syngeneically and nontransplanted lungs.

The histogram of the cell density of the BALT in the allografts shows a shift to the left and it lacks columns representing areas of high cell density (* = $p<0.05$). The percentage of squares containing 0 to 4 and 5 to 9 lymphocytes is significantly higher (* = $p<0.05$) in the allografts than in the other lungs.

In the contralateral, right, nontransplanted lungs of all groupsthe cell density of the BALT is similar.

Left lungs: dark shaded columns.
Right lungs: light shaded columns.

3.5 Defective BALT in lung allografts

High Endothelial Venules

The number of HEV in the BALT was significantly lower ($p<0.01$) in sections of the allogeneically transplanted lungs than in sections of the syngeneically transplanted and nontransplanted lungs (fig 4). Very few, or none at all, HEV were found in the allogeneic lung transplants. Also, when calculated per surface area of BALT there were only 5.0 HEV/mm^2 in the allografted lungs compared to 13.5 and 15.5 HEV/mm^2 in the syngeneic and normal lungs, respectively. The number of HEV in the BALT of the right nontransplanted lungs in the allografted rats was normal.

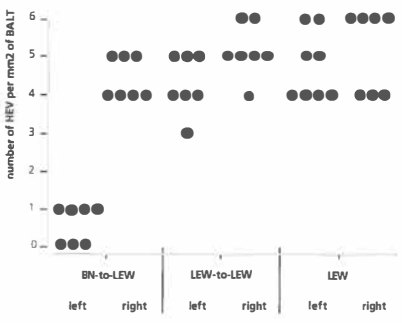


Figure 4. Number of high endothelial venules (HEV) in BALT per lung section. HEV were detected with the monoclonal antibody HIS22. Each dot represents the value of one rat.

The number of HEV was significantly lower in the BALT of the allografts than in the other lungs ($p<0.05$).

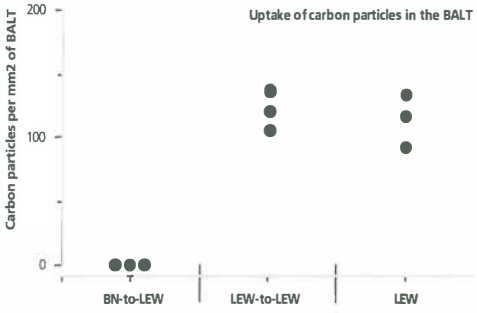
The number of HEV in the right, nontransplanted, lungs in the allografted rats was normal.

Uptake of carbon particles

Uptake of carbon particles was disturbed in the BALT of the allogeneic lung transplants (fig 5). Twentyfour hours after instillation there were no carbon particles at all in the BALT of the allogeneic lung transplants, whereas in the BALT of the other lungs between 100 and 150 carbon particles were present per mm^2 of BALT. In these lungs some carbon particles were found in the epithelium covering the BALT, but the bulk of the particles was scatteredly distributed over the whole BALT, without preferential location.

Figure 5. Uptake of carbon particles in the BALT of left lungs.
Carbon particles were administered directly into the left lung. Twentyfour hours later the number of carbon particles in the BALT were counted. Each dot represents the value of one rat.

No carbon particles could be detected in the BALT of the allografts, whereas in the BALT of the isografts and nontransplanted lungs between 100 and 150 carbon particles per mm2 of BALT were present



Lymphocyte migration

Fitc-labeled lymphocytes could clearly be detected by light microspcopy in the BALT of all lungs in the 3 groups, twentyfour hours after injection (Fig 6A and6B).

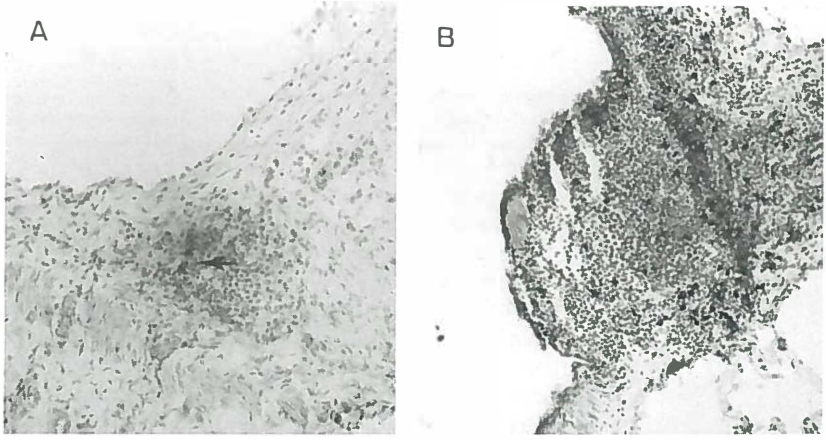


Figure 6. Migration of FITC-labeled lymphocytes into the BALT of allogeneically transplanted (A) and syngeneically transplanted lungs (B).
Twentyfour hours after injection of FITC-labeled lymphocytes, crysostat sections of the lungs were stained by immunoperoxidase for FITC-labeled lymphocytes (dark dots). FITC-lymphocytes could be detected in all lung sections investigated. Original magnification x 60.

A. Allogeneic transplants. Only few FITC-labeled lymphocytes could be detected in the remnants of the BALT (arrow).

B. Syngeneic transplants. Large numbers of FITC-labeled lymphocytes could be found all over the BALT.

The migration pattern of FITC-labeled lymphocytes into the BALT of the allogeneically transplanted lungs was different from the migration pattern into the BALT of the syngeneically transplanted and nontransplanted lungs both when determined as 'absolute' numbers and as 'relative' numbers.

The 'absolute' number of immigrating FITC-labeled lymphocytes per mm^2 of BALT in the allogeneically transplanted lungs was significantly lower ($p<0.01$) than in the syngeneically transplanted and nontransplanted lungs (fig 7).

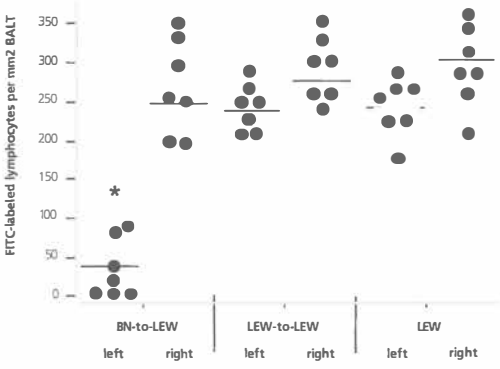


Figure 7. "Absolute" number of FITC-labeled lymphocytes per mm2 BALT.
Each dot represents the value of one lung. The horizontal lines represent the average values.

In the allografts the "absolute" number of immigrating lymphocytes was significantly lower than in the isografts and nontransplanted lungs (* = $p<0.01$).

In the contralateral, right, nontransplanted lungs in the allografted group number of immigrating lymphocytes in the BALT was normal.

Immigrating lymphocytes in the BALT of the allogeneically transplanted lungs were only present in those areas of BALT with an almost normal cell density, whereas they were present throughout the BALT of the syngeneically transplanted and nontransplanted lungs. In the contra-lateral right lung of the allogeneically transplanted group the 'absolute' number of immigrating lymphocytes into the BALT was normal.

The 'relative' number of FITC- lymphocytes in the selected areas in the BALT of the allogeneically transplanted lungs with highest cell density was close to that in the BALT of the syngeneically transplanted and nontransplanted lungs (fig 8).

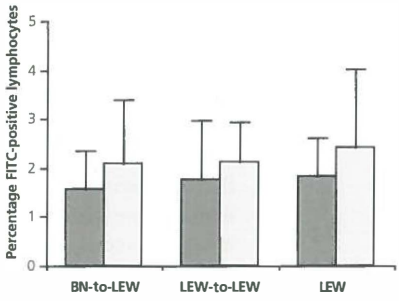


Figure 8. Relative number of FITC-labeled lymphocytes in areas of BALT with normal cell density.
In the selected areas of the BALT in the allografts with the hihgest cell density, the precentage of FITC-labeled lymphocytes was close to that in the BALT of the isografts and nontransplanted lungs.

Left lungs: dark gray shaded colums.
Right lungs: light gray shaded columns.

Discussion

This study shows that BALT in long-term surviving rat lung allografts is defective in all three investigated aspects: its structure, uptake of particles and the migration of lymphocytes. In contrast, BALT in the syngeneically transplanted lungs was normal in all these aspects. Our discussion of these changes in the BALT of lung allografts will emphasize on its consequence for the function of the BALT in the local antibody response in the lung.

The structure of the BALT is changed after lung allotransplantation. Whereas BALT in the normal lung consists of a dense collection of lymphoid cells closely associated with the bronchial epithelium, in long-term surviving rat lung allografts it is reduced in size and cell density and is replaced by fibrous tissue. The fibrous tissue isolates the BALT from the bronchial epithelium. Changes in the structure of the BALT in allografted lungs have implications for the function of the BALT. First, antigen uptake from the airways into the BALT requires a close relationship with the airways (5). Second, to generate an approriate antibody response sufficient lymphoid cells have to be present in the BALT and lymphocytes should be able to migrate into the BALT for adequate immune surveillance (4,5,7,8). These two processes are affected by the structural changes, as was shown by the experiments on particle uptake and lymphocyte migration.

3.5 Defective BALT in lung allografts

The prominent presence of fibrous tissue in the BALT of long-term surviving rat lung allografts disturbes the uptake of antigens into the BALT. Twentyfour hours after intrabronchial administration of carbon particles no carbon particles were found in the BALT of allogeneically transplanted lungs whereas in the BALT of the syngeneically transplanted and nontransplanted lungs carbon particles were scattered throughout the BALT. Because in the allogeneically transplanted lungs the uptake of antigens, at least particulate antigens, from the airways is prevented they cannot be presented to the lymphoid cells in the BALT; hence, an appropriate antibody response will not be initatiated in the BALT.

Changes in structure of the BALT also disturbed the lymphocyte migration into the BALT of rat lung allografts. In the allografted lungs the 'absolute' number of immigrating FITC-labeled lymphocytes in the BALT was reduced enormously. Two explanations are conceivable for this reduced migration. First, we found in this study that the reduced lymphocyte migration corresponded with a low number of HEVs in the BALT of the lung allografts. Because HEVs in the BALT are the only entry-site for lymphocytes from the circulation, disappearance of HEVs will reduce migration of lymphocytes to the BALT. Since the number of HEVs is controlled by the amount of antigenic stimulation (8) it is conceivable that disappearance of HEVs is secundary to a reduced antigenic stimulation of the BALT. Because all animals were housed under the same experimental conditions, we presume that the antigenic stimulation of the BALT in the allogeneic lung transplants is decreased as a result of the fibrous layer between the epithelium and the BALT preventing uptake of antigens. A second explanation for the reduced migration of lymphocytes to the BALT might be the inability of recipient lymphocytes to enter the allogeneic environment of the lung allograft. This explanation would be in concert with Lear et al. who found that in small bowel transplants recipient lymphocytes did not repopulate the allogeneic bowels' local lymphoid tissue (the Peyers' Patches) (15). They

suggested that the inabiltity of recipient lymphocytes to enter the donor lymphoid tissue of the transplanted small bowel prevented the repopulation of the lymphoid tissue. Also another report suggested that normal migration of recipient lymphocytes into transplanted organs is changed after allogeneic transplantation (16). However, our study does not support this explanation because we found almost normal 'relative' numbers of FITC-labeled lymphocytes in the areas of the allogeneic BALT with normal cell numbers. This indicates that the turnover of lymphocytes in these undamaged areas is normal and thus that recipient lymphocytes are able to enter the allogeneic environment of the donor lymphoid tissue in the BALT of the lung transplant. The migration to the cell poor areas of the allogeneic BALT is probably largely affected by the fact that the fibrous tissue has replaced most of the lymphoid tissue. Taken together, our findings indicate that migration of lymphocytes to the BALT of the allogeneic lung transplants is reduced as a result of fibrotic changes in the subepithelial area and in the lymphoid tissue itself.

The damage of the BALT in the lung lung transplants is not caused by the transplantation procedure, but is most likely caused by rejection. This is concluded from our finding that the BALT in the syngeneically transplanted lung, in contrast to BALT in the allogeneically transplanted lung, was normal without any fibrotic changes. Already in earlier reports it was suggested that the BALT is a target of rejection after allogeneic lung transplantation (9). In untreated rat lung transplants, recipient lymphocytes were shown to infiltrate the BALT early in the rejection process and to cause a mixed lymphocyte reaction that destroys the donor lymphocytes in the BALT (9). After cyclosporine treatment the lungs survive indefinately, as in our present study, but also under this condition a chronic rejection process may preferentially damage the BALT. Similarly, Westra et al found that after combined heart-spleen transplantation the lymphoid tissue was damaged specifically (17). In CsA treated rats, the hearts survived indefinately, but the concomitantly transplanted spleens were damaged. They suggested that the spleen filters

anti-graft reactive lymphocytes out of the circulation, so that these cells do not reach the heart. It is conceivable that a similar mechanism is responsible for the survival of lung allografts after cyclosporine treatment. In this way anti-graft reactive lymphocytes accumulate in the BALT, which is subsequently damaged by a local rejection process, despite CsA immunosuppression. It is obvious that, like in the combined heart-spleen transplantation, this local rejection process does not proceed to rejection of the lung parenchyma. The BALT of the transplanted lung is damaged by a chronic rather than an acute rejection process and replaced by fibrous tissue. An identical replacement of the Peyers' patches by fibrous tissue was found in long-term surviving rat small bowel transplants with chronic rejection (18). Similarly, a clinical study reported a depletion of BALT in two lung transplant recipients in association with chronic rejection (19).

Extrapolation to human lung transplantation, however, is not immediately possible. In humans the BALT is not a similar constituent part of the lung as it is of rat lungs (5,20). Nevertheless, the human lung also contains an extensive lymphoid system although not always structured in dense aggregates like BALT. Furthermore, it is hard to investigate whether infection incidence after clinical lung transplantation increases as a result of a damage of the local defense system. All patients receive immunosuppressive agents that makes them more susceptible for infection. Comparing lung transplant recipients with recipients of other organ grafts, however, shows that they have many infection episodes: for lung transplant recipients 3.36 episodes per patient in the first postoperative year compared to 1.41 and 1.83 episodes for heart and liver recipients, respectively (21). Based on this comparison it is not unlikely that the defense in the transplanted lungs is impaired in humans as it is in rats. At least, there is evidence that the number of peribronchial plasma cells as a possible equivalent of BALT is reduced in human lung transplants (19).

Conclusion

Our study demonstrates that after allogeneic transplantation the BALT in the lung is defective in respect of structure and function, probably as a result of a chronic rejection process. In this defective BALT an antigen can not initiate an adequate antibody response so that it will be cleared slowly from the transplant. Exactly this was observed during viral infection of airways in rat lung allografts. The airway infection did not induce an antibody response in the BALT (3), and the virus remained present for a longer time in these allografts than in normal lungs, causing severe and permanent airway damage (22). It is likely that also in patients the BALT in the transplanted lung is defective after lung transplantation (19), which may explain the high incidence of infections after lung transplantation. In the view that rejection is probably the primary cause of the structural damage of the BALT, we speculate that in the future better immunosuppressive regimens will preserve the BALT in the transplanted lung and leave the local immune system intact.

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3.5 Defective BALT in lung allografts

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4 Immune responses in human lung transplants

4.1 Detection of immune responses in human lung transplants

As mentioned in previous chapters, bronchiolitis obliterans (BO) is the major long-term complication of lung transplant recipients. BO is characterized by a slowly progressive obstructive airway disease caused by fibrous obliteration of bronchioles and bronchi, resulting in an irreversible decline of lung function (1,2). Development of BO is by no means specific to transplanted lungs but also occurs after a variety of respiratory infections (3) and autoimmune disorders (4). As reviewed in chapter 1.2, the cause of the initial injury of the bronchiolar epithelium and the subsequent immunological mechanisms that ultimately lead to bronchiolitis obliterans in human lung transplants are still not fully understood. In chapters 2 and 3 of this thesis we have demonstrated in our rat model of lung transplantation that BO is caused by a derailment of immune and inflammatory responses induced by chronic rejection and infection in these lung transplants. We presume that in human lung transplant recipients BO is caused by similar mechanisms of derailed immune and inflammatory responses as we have found in rats. Analysis of these immune and inflammatory responses in human lung transplants would help to gain insight in the mechanisms that lead to bronchiolitis obliterans in these lung transplants and more importantly, might improve early detection of these responses in the lung transplant. That would enable logical and timely therapeutic approaches and consequently prevention of development of BO.

Analysis of the immunological responses and pathological events in the lung transplant that lead to BO can be made most reliably by investigation of cells or tissue obtained from the transplanted lung itsself. Bronchoalveolar lavage (BAL) of cells from the alveolar spaces of the transplanted lung offers a method for monitoring of the cells that are involved in (chronic) rejection and infection (5, 6, 7). Although BAL cells can have alloreactive properties no clear correlation between the characteristics of BAL cells and histological findings could be demonstrated during acute and chronic lung rejection in experimental and clinical studies (7, 8). One of the reasons for this poor correlation could be that pre-alveolar airways are the

main site of origin of the cells that are sampled by BAL (8), so more proximal airways and lung tissue are not sampled. Nevertheless, BAL cytology and culture have an established role in the diagnosis of opportunistic pulmonary infections, both in nontransplant patients and lung transplant recipients (8, 9).

Tissue specimens for immunological and histological examination of the transplanted lung can be obtained by biopsy. Basically, two types of lung biopsy can be distinguished: open lung biopsy and transbronchial biopsy (TBB). In the early years of lung transplantation open lung biopsy was used to make a definitive diagnosis of rejection (10). Open lung biopsy, however, needs a thoracic operation with the risk of bleeding in the pleural adhesions induced by previous operations (including the lung transplantation itsself). Furthermore, this technique cannot be used frequently and, because of the associated risks, cannot be jusitified in asymptomatic patients. The technique of TBBs is less invasive then open lung biopsies and can be performed serially (11,12). TBBs are performed through a fiberoptic bronchoscope under mild sedation of the patient. A forceps is positioned in the periphery of the lung where the biopsy is taken. With this technique it is usually possible to obtain lung tissue containing the three main pulmonary structures: bronchiolar epithelium, alveolar tissue and blood vessels (figure 1) (13).

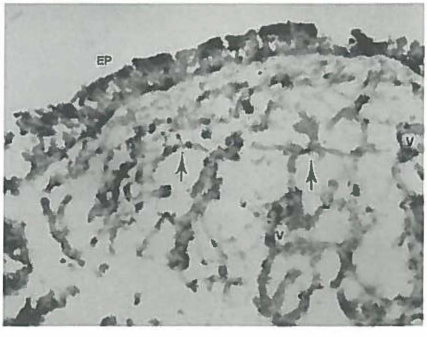

Figure 1. TBB specimen.
Example of fresh frozen lung tissue obtained by transbronchial biopsy.

In this TBB speciment all three lung structures are present: epithelium (EP), blood vessels (V) and alveolar walls (arrows)

In this particular specimen T cells are detected with a monoclonal antibody (darkly stained cells).

TBBs are valuable in the diagnosis of certain forms of diffuse lung disease, for example sarcoidosis (14), and was found useful in early reports of opportunistic infections in immunocompromised patients (15).

One of the drawbacks of TBBs is the relatively small size of the lung tissue in each biopsy. Even the largest TBB speciment contains little lung tissue generally providing alveolar tissue, a few vessels and only rarely airways for evaluation (13). In addition, lung rejection in its onset is not a diffuse but a focal process and it does not affect all structures simultaneously (16, 17, 18). As a consequence, it is not uncommon that a TBB specimen fails to show histopathologic abnormalities during early acute or chronic lung rejection (19). Because of this sampling error, the early experience of TBBs in lung transplantation was not encouraging (20).

Despite initial reluctance to try transbronchial lung biopsy in heart-lung transplant recipients, the Papworth group in 1988 reported their experience using TBBs in the diagnosis and management of lung allograft rejection (21). In order to reduce the possible sampling error TBBs were taken under fluoroscopic control, guiding a large alligator forceps to areas of pulmonary shadowing (figure 2).

Figure 2. Obtaining of TBB. Under fluoroscopic controle a large alligator forceps is directed to an area of pulmonary shadowing (asterisk) in a transplanted lung.

In addition, three or four biopsies were taken from each lobe of one lung, even when the lobes did not show any signs of shadowing. With this rigorous protocol an overall sensitivity for the detection of acute rejection of 84% was reached and a specificity of 100% (19, 21). Since 1988 most lung transplant centers follow the Papworth experience by monitoring of the lung transplant by TBBs (22-27) and to date TBBs are still considered as the gold standard for monitoring of both acute and chronic rejection . This is further emphasized by the establishment of an uniform pathological grading system for pulmonary rejection in TBBs under the supervision of the International Society for Heart and Lung Transplantation (28).

Since 1988 numerous reports about the use of TBBs in the management of pulmonary rejection have been published (19, 22-31). Most of these reports, however, describe the histopathological findings in TBBs during the different stages of acute and chronic rejection. Although some elegant long-term serial TBB studies in heart-lung transplant recipients have been performed on the development of bronchiolitis obliterans in lung transplants (19, 22, 30, 31), our knowledge of the immunological mechanisms leading to bronchiolitis obliterans is still poor. Since TBBs provide lung tissue exactly from the place were the actual immunological and inflammatory responses occur during acute and chronic rejection and during infection, analysis of cell types infiltrating the lung tissue during these processes could increase our knowledge about the mechanisms involved in the development of bronchiolitis obliterans. Furthermore, analysis of these on-site responses would enable a comparison between experimental and clinical findings. As a first step to analyse the immune and inflammatory responses in human lung transplants, we used immunohistochemical phenotyping of infiltrating cells during acute and chronic rejection in human heart-lung transplants, as described in the next chapter.

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4.2 Distinct phenotypes of infiltrating cells during acute and chronic lung rejection in human heart-lung transplants

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4.2 Infiltrating cells during acute and chronic lung rejection in humans

Abstract

To differentiate between acute and chronic lung rejection in an early stage phenotypes of infiltrating inflammatory cells were analysed in 34 transbronchial biopsies (TBBs) of 24 patients after heart-lung transplantation. Transbronchial biopsies were taken during during acute lung rejection and chronic lung rejection, as diagnosed by clinical data and histopathological investigation. TBBs without rejection and normal lung tissue specimens served as controls.

Distinct phenotypes of inflammatory cells were found in acute and chronic lung rejection. T cells were present both in acute and in chronic rejection, but did not differentiate between them. In contrast, B cells with antibody deposition were mainly present in chronic rejection and not in acute rejection. Activated macrophages were only present in acute rejection and not in chronic rejection. In nonrejecting lung transplants perivascular infiltrating cells were virtually absent. In the biopsy specimen, vessels had to be available for analysis, because the cell phenotypes were best recognised in perivascular infiltrates.

The analysis of specific phenotypes of inflammatory cells by immunohistochemistry supports the diagnosis of acute and chronic lung rejection, in particular in those cases in which transbronchial biopsy provides limited tissue without airways.

Introduction

Heart-lung transplantation is a treatment for patients with end-stage lung disease or lung vascular disease. Over the last decade the survival of transplanted patients and their quality of life have improved, but are still behind compared to those of patients with other solid organ transplants. An important cause of mortality and morbidity after lung transplantation has always been and still is rejection (1,2). In the period before 1980, acute lung rejection was a serious complication. Nowadays, chronic rejection has emerged as a complication in half of the long-surviving patients (3,4,5). Adequate and timely treatment of rejection heavily depends on methods for early diagnosis of rejection.

In diagnosing acute and chronic rejection, transbronchial biopsies (TBBs) have proven to be very helpful. The introduction of TBBs has made it possible to obtain graft specimens as a routine procedure in the management of patients after lung transplantation (6,7). The TBB specimens usually allow classification of the histopathological findings of

rejection according to a uniform grading system of the International Society for Heart and Lung Transplantation (8). This grading system divides acute rejection into 5 grades: no, minimal, mild, intermediate and severe acute rejection, and recognises 4 categories of chronic rejection and inflammation, describing airways and vessels separately.

The accurate assignment of histopathological findings to a rejection grade is easily biased by sampling error of the biopsies (9,10). One has to realise that even the largest TBB specimen contains little lung tissue, generally providing alveolar tissue, a few vessels and only rarely airways for evaluation. In addition, lung rejection in its onset is a focal process and it does not affect all structures simultaneously (11,12). As a consequence, it is not uncommon that a TBB specimen fails to show histopathologic abnormalities during early acute or chronic rejection (13,14). To reduce the sampling error, several biopsies are to be taken in each case. In an experimental study a total of 5 specimens were needed to achieve a 92 % sensitivity during mild acute rejection (15). In Cambridge, we prefer to take 3 TBBs from each lobe of a lung transplant. With this protocol we found that recognition of acute and chronic rejection in TBB specimens is sensitive and highly specific (9,10).

A different approach to reduce the influence of a sampling error might be to increase the sensitivity of the histological evaluation. We think that this might be achieved by immunohistochemical phenotyping of infiltrating cells in TBBs. Such an analysis of biopsies of heart (16,17), kidney (1819,20) and liver transplants (21) showed specific infiltration patterns during acute and chronic rejection. Up to now, experience with immunohistochemical phenotyping of infiltrating cells in TBB specimens during acute and chronic lung rejection is very limited. Only one recent study described the phenotypes of infiltrating cells in TBBs during acute rejection (22). Furthermore, phenotypes of infiltrating cells during chronic lung rejection have been analysed in only 4 patients with OB, being the end-stage of a presumed chronic rejection process (23,24).

4.2 Infiltrating cells during acute and chronic lung rejection in humans

The present study was designed to analyse the phenotypes of infiltrating cells in TBBs during mild acute and chronic lung rejection. Two questions were addressed. First, whether infiltrating cells with specific phenotypes were indicative of acute or chronic rejection in an early stage. And second, which lung structures (vessels, airways, alveolar walls) had to be available in the TBB specimens for analysis of these cells. Therefore, we investigated phenotypes of infiltrating cells in TBBs from patients with mild acute rejection (AR), chronic rejection (CR) and without rejection (NR) as diagnosed by clinical data and by routine histopathological investigation of several TBBs, and cells in normal lung tissue (NL).

Materials and methods

Patients

We analysed 34 TBBs of 24 heart-lung transplant patients transplanted at Papworth Hospital. Postoperatively all patients received cyclosporine at a dose adjusted to maintain whole blood levels between 300 and 500 ng/L and oral azathioprine, in a dosage to keep the total white cell count less than 6×10^9 /L.

TBBs

TBBs were performed routinely at 3 and 6 months and then annually after surgery. Also, TBBs were performed when lung rejection or infection was suspected based on clinical symptoms, even in the presence of a normal appearing radiograph. The TBB procedure was performed through a fiberoptic bronchoscope under fluorscopic control. Three biopsies were taken from each lobe of one lung.

All routine biopsies were used for histopathological investigation. They were fixed in neutral buffered formalin, embedded in paraffin wax, and sections were stained with hematoxilin & eosin, Pearls' elastic van Gieson, methyl green pyronine, Grocott-Gomori methenamine-silver nitrate, and periodic acid-Schiff stains. The sections were read for evaluation and graded according to the grading system of the International Society for Heart and Lung Transplantation (8). Rejection grades relevant for this study are explained in table 1.

Inclusion of patients

During each routine TBB procedure one extra biopsy was taken for immunohistochemical analysis of infiltrating cells. In this study we included TBBs from patients that at the time of biopsy were assigned to the mild AR group, active CR group or NR group. Assignment to these groups was based on the evaluation of multiple routine TBBs and confirmed by the clinical course and response to treatment. Patients were only included in this study when infection could be excluded based on clinical and laboratory data.

Of the 34 TBBs, 13 were assigned to the AR group, 14 to the CR group and 5 to the NR group. Details are given in table 1. In addition, 2 follow-up biopsies were analysed from 2 of the patients in the CR group when they had inactive total bronchiolitis obliterans (grade C2b).

As a control group in this study we used normal lung tissue (NL) obtained from 10 patients undergoing pneumonectomy or lobectomy. None of the patients had evidence of lung infection. No patient was taking corticosteroids or other immunosuppressive agents, and none of the patients had smoked within 6 months before surgery. Tissue samples were taken from macroscopically normal lobes. On histological examination the tissue samples showed no evidence of inflammatory changes: there were no signs of perivascular infiltration, nor an increase of lymphocytes in the alveolar region.

Monoclonal antibodies

Monoclonal antibodies reacting with specific cell phenotypes were used in a two step immunoperoxidase technique or in an indirect immunofluorescence technique on cryostat sections. The monoclonal antibodies recognised CD2 (T cells), CD4 (T helper cells), CD8 (T cytotoxic cells), CD25 (Interleukine-2 [IL-2] receptor), CD20/22 (B cells), IgM and IgG depositions (indirect immunofluorescence) and CD14 (activated macrophages).

Biopsies for immunohistochemical staining were frozen in freon (- 90^0 C) and stored at - 80^0 C. Serial cryostat sections (4 µm) were air-dried (20 min) and fixed in acetone (10 min) at room temperature.

Peroxidase staining

The sections were rinsed 3 times in phosphate-buffered saline (PBS), then incubated with 25-50 µL of the monoclonal reagent, depending on the section's size in the optimal dilutions, for 60 min at room temperature. Negative controls were incubated in PBS as a negative control. After rinsing in PBS, peroxidase-conjugated rabbit anti-mouse Ig serum (Dakopatts, Copenhagen, Denmark) was applied for 30 min in a dilution of 1:20 at room temperature, the incubation medium being supplemented with 1% human AB serum. For the peroxidase reaction, the sections were incubated in 3-amino-9-ethylcarbazole and H_2O_2 for 10 min. Nuclear counterstaining was performed with Haemalum, and the sections were mounted with Kaiser's glycerin-gelatin.

Immuno-fluorescence

The sections were rinsed 3 times in PBS and were incubated with unconjugated antibodies against human IgM and IgG and with mouse immunoglobulin (Cooper Development Company, Malvern, USA) as negative control. All incubations were performed for 30 minutes at room temperature, then rinsed 3 times in PBS and then stained with fluoresceinated rabbit-antimouse IgG in an indirect immunofluorescent method for 30 minutes. After the incubations slides were covered with polyvinyl alcohol and were examined immediately with a fluorescent microscope. All slides with positive staining were photographed.

Cell scores

Assessment of the staining results was done by two observers (JBW and ASHG) without knowledge of the clinical diagnosis. At least three fields in each TBB were examined at a magnification x60. The numbers of positively stained cells were scored recognising 4 cell scores in which - = no cells, + = low (1-30 cells per 3 fields), ++ = intermediate (30-75 cells per 3 fields) and +++ = high (> 75 cells per 3 fields) numbers of positive cells. Cell scores of JBW and AG were similar, with a correlation coefficient r of 0,79 ($p<0.01$). After scoring of the TBB specimens the histological diagnosis of NL, NR, AR or CR was disclosed and the results were grouped accordingly.

Statistical evaluation

The number of biopsies with positive and negative staining were compared with the Fischer's exact test. Cell scores, time of TBB after HLT were compared with the Mann-Whitney rank sum test. Correlation of cell scores in the perivascular and peribronchial regions was performed with the Spearman rank correlation coefficient. All statistical calculations were performed with the statistical software package Statview II™ for the Apple Macintosh computer. In all tests p values of less than 0.05 were considered statistically significant.

Table 1.

Group	N	Rejection classification	Time of TBB* in months (range)
NR	5	grade A0	8.4±4.5 (6-14)
AR	13	grade A2a (n=7), grade A2b (n=6)	3.0±2.2 (1-14)
CR	14@	grade C1a (n=14) with grade D (n=6)	26.0±15.3 (3-56)**

Classification of lung rejection of the International Society for Heart and Lung Transplantation

Grade A0	**no significant abnormality**
Grade A2a	**mild acute rejection, with evidence of bronchiolar inflammation**
Grade A2b	**mild acute rejection, without evidence of bronchiolar inflammation**
Grade C1a	**chronic airway rejection, active subtotal bronchiiolitis obliterans**
Grade C2b	**chronic airway rejection, inactive total bronchiolitis obliterans**
Grade D	**chronic vascular rejection**

* mean time of TBB (and range) after HLT, @ In addition, 2 follow-up biopsies were obtained after bronchiolitis obliterans had developed, grade C2b, ** CR significantly later than AR, $p<0.05$

Results

Of the three main lung structures, blood vessels, airways and alveolar walls, only vessels and alveolar walls were present in all TBBs and could be fully investigated for analysis of cell phenotypes. In contrast, bronchioles were only present in 15 of 34 (44%) TBBs; in 7 TBBs with AR, 6 with CR and 2 with NR. Because of this small number of bronchioles we don't present the full analysis of the infiltrating cells in the peribronchial region, but only the correlation of T and B cell numbers with those in the perivascular region. We present the results of phenotyping analysis of T cells and subsets, IL2-receptor positive cells, B cells, Ig depositions and activated macrophages in this order. For each phenotype we compare the NL group with the AR and CR groups, respectively. Since the number of TBBs in the NR group and the cell phenotypes are identical to the those in the NL group, the results of this group is not listed separately in the tables and are not used for statistical analysis.

T cells

Table 2.

In the perivascular region of the *normal and nonrejecting lung* T cells were absent in all tissue specimens (fig 1A). During *acute rejection,* T cell scores in the perivascular region were increased to low or intermediate in all TBBS (fig 1B) ($p<0.05$ vs NL). Among T cells, CD4-positive cells and CD8-positive cells were present in equal numbers in each TBB. During *chronic rejection,* T cell scores were increased in all TBBs, ($p<0.05$), like in AR. CD4-positive and CD8-positive cell numbers were equal in most TBBs.

Peribronchially, T cells were present in all TBBs of the AR (fig 1C) and CR groups with bronchioles. The T cell scores in the peribronchial region correlated closely with those in the perivascular region (table 4, correlation coefficient 0.83, $p<0,001$). The T cell scores of the peribronchial region were lower than those of the perivascular region ($p<0.001$).

In the alveolar walls of the *normal and nonrejecting lung* and the, T cell scores were low to intermediate in all specimens. Among T cells, CD4-positive were less numerous than CD8-positive cells ($p<0.05$). In *acute rejection* (fig 1B) and in *chronic rejection* the total T cell scores in alveolar walls were not increased compared to NL and NR.

Table 2. T cells and T cell subsets, distributed by cell scores

Group	perivascular region				alveolar walls			
	-	+	++	+++	-	+	++	+++
CD2 - all T cells								
NL	10	-	-	-	-	8	2	-
AR	-	2	3	8*	-	10	3	-
CR	-	1	7	6*	-	9	5	-
CD4 - helper/inducer T cells								
NL	10	-	-	-	-	7	3	-
AR	2	2	6	3*	-	8	5	-
CR	3	3	6	2*	-	10	4	-
CD8 - cytotoxic/suppressor T cells								
NL	10	-	-	-	-	7	3	-
AR	1	2	8	2*	-	9	4	-
CR	2	1	9	2*	-	11	3	-

* = p<0.05 compared to NL

Table 3. Correlation of infiltration scores in the perivascular and peribronchial regions in TBBs with both regions present.

Vessels and bronchiolar tissue were only present in 13 of 29 TBBs (45%); 7 TBBs with AR and 6 with CR.
T cells were present during AR and CR, B cells only during CR and macrophages only during AR.
Rho is Spearman's rank correlation coefficient corrected for ties.

Cell type	N	Rho	p
T cells	13	0.83	<0.001
B cells	6	0.74	<0.01
Macrophages	7	0.78	<0.01

4.2 Infiltrating cells during acute and chronic lung rejection in humans

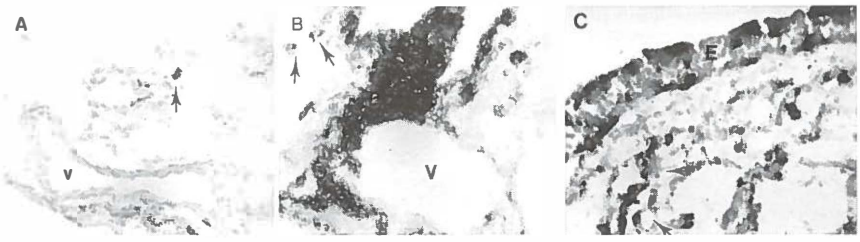

Figure 1. T cells in normal lung (A) and during acute rejection in lung tranplants (B and C). Cryostat sections of lung biopsies were stained with immunoperoxidase for CD2 positive T cells (original magnification A and B x160; C x210).

1A. Normal lung speciment with vessel (V) and alveolar walls (arrow heads). No T cells are seen in de perivascular region. In the alveolar walls of this biopsy T cells are present in low numbers.
1B. TBB of lung transplant during acute rejection. Intermediate numbers of T cells are present in the perivascular region. In the alveolar walls T cell numbers are low.
1C. Same TBB as 1B, showing bronchiolar tissue. T cells are present in the epithelium and in the submucosa, but they are less numerous than in the perivascular region as shown in 1B

IL2-receptor IL2-receptor-positive cells were invariably absent in all specimens of the NL, NR, AR and CR groups in the perivascular and peribronchial region and in the alveolar walls.

B cells
Table 4. In the perivascular region of the *normal and nonrejecting lung*, B cells were absent in all lung specimens. Also in *acute rejection* perivascular B cells were absent in all TBBs, even in those with intermediate or high T cell scores. Surprisingly, in *chronic rejection*, B cells were found in 12 of 14 TBBS in intermediate numbers (fig 2A). These B cells distinguished the CR group not only from NL but also from the AR group ($p<0.01$). The 2 additional TBBs from patients with bronchiolitis obliterans showed no perivascular B cells.

Peribronchially, the correlation of B cells in the peribronchial and perivascular region was as remarkable as that of T cells (table 4, correlation coefficient 0.74, $p<0.01$). B cells were only present in the biopsies of patients in the CR group. Like T cells, the B cell scores were lower in the peribronchial region than in the perivascular region ($p<0.001$).

In the alveolar walls of the *normal and nonrejecting lung*, B cells were not present. In *acute rejection* , B cells were absent in most TBBs. In *chronic rejection*, B cells were present in almost half of the TBBs. Their numbers were significantly higher than in NL ($p<0.05$), but the difference was less striking than in the perivascular region.

Table 4. B cells, distributed by cell scores

Group	perivascular region				alveolar walls			
	-	+	++	+++	-	+	++	+++
CD20/22 - all B cells								
NL	10	-	-	-	10	-	-	-
AR	12	1	-	-	10	3	-	-
CR	2	1	11	1*	8	-	6	-**

* = $p<0.01$ compared to NL and AR, ** = $p<0.05$ compared to NL and AR

IgM and IgG In the *normal and nonrejecting lung* and during *acute rejection* there were no IgM and IgG depositions in the vessel wall. Only during *chronic rejection* granular immunoglobulin deposition could be found in TBBs (fig 2B). IgM deposition was obvious in the vessel wall (8 of 14) and in the bronchiolar wall (4 of 6) (both $p<0.05$ compared to NL). IgG deposition was less pronounced: in the vessel wall (4 of 14) and in the bronchiolar wall (1 of 6) which was not significantly different from NL. In the alveolar walls IgM and IgG depositions were less significant (4 and 3, respectively). The 2 TBBs with bronchiolitis obliterans showed no immunoglobulin deposition in any of the structures.

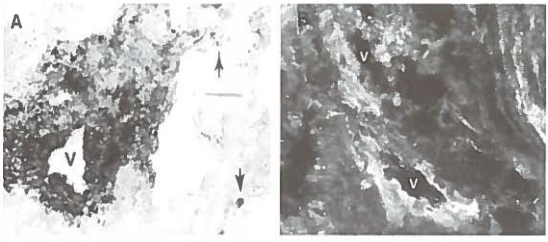

Figure 2. B cells (A) and IgM depositions (B) during chronic rejection.
Sections are from the same biopsy, magnification x160.

2A. Many B cells are present around the vessel (V).
2B. Strong fluorescence for IgM in the perivascular region.

Macrophages In the perivascular region of the *normal and nonrejecting lung*, CD14-positive macrophages were absent. In *acute rejection*, CD14-positive macrophages reached intermediate scores in perivascular infiltrates in most TBBs ($p<0.01$ compared to NL). In *chronic rejection*, CD14-positive macrophages were present in few TBBs, which was not significantly different from NL.

Table 5.

Peribronchially, the correlation of CD14-positive macrophages in the peribronchial and perivascular region was similar to the correlation for T and B cells (table 4, correlation coefficient 0.78, $p<0.01$). CD14-positive macrophages were only present in the TBBs of patients with AR.

Table 5. Activated macrophages, distributed by cell scores

Group	perivascular region				alveolar walls			
	-	+	++	+++	-	+	++	+++
WT14 - activated macrophages								
NL	10	-	-	-	8	2	-	-
AR	3	-	10	-*	9	-	4	-
CR	12	-	2	-	12	2	-	-

* = $p<0.05$ compared to NL and CR

In the alveolar walls, CD14-positive macrophages were not increased significantly in any of the groups, although they were found in 4 of 13 TBBs during AR.

Discussion

The first conclusion from this study is that infiltrating cells with distinct phenotypes, being T cells, B cells and activated macrophages, are indicative of acute and chronic rejection after human heart-lung transplantation.

The appearance of T cells in TBBs is an indication of lung rejection, but it does not differentiate between acute and chronic rejection: the scores of total T cells, CD4-positive (T helper) and CD8-positive (T cytotoxic/suppressor) cells in perivascular infiltrates were equally high during acute and chronic rejection. This is in concert with observations in heart (16,17), kidney (18,19) and liver transplants (21,25) where T cell infiltrates of CD4-positive and CD8-positive cells were found during both acute and chronic rejection.

The IL-2 receptor on activated lymphocytes is of no value for phenotyping of cells during rejection, in fact it could not be detected in any of the rejection groups. Other investigators also failed to find IL-2 receptor expression in biopsies during

acute heart and liver rejection (25,26), possibly because this receptor is expressed only briefly and early during lymphocyte activation.

B cells and vascular antibody depositions in TBBs are indicators of chronic rejection: these were mainly present during chronic rejection and not during acute rejection. However, since most of the TBBs in the CR group were taken significantly later after transplantation than those in the AR group, presence of B cells could also just be an epiphenomenon of time. Although in this study we cannot definitely exclude this possible role of time after transplantation, B cells were absent in the 5 TBBs without rejection that were taken significantly later the the TBBs in the acute rejection group (table 1). In addition, our observation of antibodies in the CR group is in agreement with other studies on chronic rejection in other transplants, such as heart-lung (27), heart (28,29) and kidney (30). In few studies B cells have been reported to infiltrate in kidney transplants during chronic vascular rejection (30,31). In these kidneys the B cell infiltrates were mixed with T cells. Also in lung transplants, B cells were mixed with T cells in infiltrates during chronic rejection in the patients of the present study and in animals studies (32, unpublished observations). Thus, chronic rejection is characterized by a mixed infiltrate of B and T cells, often with antibody depositions.

It is worthwhile to note that B cells are involved exclusively in the early development of chronic rejection. Once bronchiolitis obliterans had developed in the 2 additionally taken follow-up TBBs, B cell infiltrates (and immunoglobulin deposition) had disappeared. This is in agreement with the report that B cells were absent in the lung tissue of a 4 patients bronchiolitis obliterans (23,24).

Activated macrophages are indicative of acute rejection: they were present in all TBBs during acute rejection, and not during chronic rejection. In several other studies, macrophages have been found during acute rejection of heart (33) and kidney transplants (20). In the present study, macrophages were identified with a new monoclonal antibody

WT14, which is directed against the CD14 cluster of monocytes and macrophages with increased affinity for activated cells. In kidney transplants WT14-positive macrophages appeared to be an excellent indicator of acute interstitial rejection (20).

The second conclusion from this study is that it is most important that vessels are available in TBBs for the phenotype analysis of infiltrating cells. In this perspective, it is fortunate that vessels are present in virtually all TBBs, together with alveolar walls. The alveolar walls have little value for the phenotype analysis because they are not infiltrated during the early stages of acute (11,12) rejection; also during chronic rejection, alveolar walls show few changes (34) except for some B cells infiltration in this study. The use of airways for phenotype analysis of infiltrating cells is restricted by the fact that they are present in a minority of the TBBs, 45% in this study. This does not form a real problem for the evaluation of TBBs, because the phenotypes of cells in peribronchial infiltrates correlate closely with those in perivascular infiltrates, both during acute and chronic rejection. And in addition to that, analysis of the perivascular infiltrates may even be more reliable because its cell scores are more pronounced. Therefore phenotype analysis of cells in the perivascular infiltrates can be considered to be representative for a given TBB.

Two aspects of this study might be argued. First, we analysed only the early stages of acute and chronic rejection, without a long-term follow-up of individual patients. The reason for this is that analysis of the phenotypes of infiltrating cells in the early stages will give most useful information to conventional histology, because in the early stages histopathological changes are most subtle and difficult to detect. A follow-up study of individual patients would be most interesting in respect of the presence of B cells in the natural history of chronic rejection. Second, there is some discrepancy between the pathological definition of chronic lung rejection, as used in this study, and the clinical definition (35,36). From the pathological viewpoint chronic lung

rejection is determined by lymphocytic bronchiolitis and submucosal fibrosis of the airways even with minimal bronchiolitis obliterans (8,10). From the clinical viewpoint chronic lung rejection is always associated with a significant, usually irreversible decline in FEV1 (35,36). We prefer the pathological definition of chronic rejection because it includes the developing process of chronic rejection before the lung function is affected. Bronchiolitis obliterans, being an ominous clinical diagnosis, is the end-stage, but not the only stage of chronic lung rejection.

This study has shown that analysis of specific phenotypes of infiltrating cells by immunohistochemistry supports the pathological diagnosis of acute and chronic lung rejection. Phenotyping will not replace conventional histopathological evaluation of TBBs, but it can give valuable additional information, in particular in those cases when TBBs provide limited tissue without airways. Therefore, we believe that phenotyping of inflammatory cells in TBBs may contribute to further improvement of the survival rate and quality of life of patients after heart-lung and lung transplantation.

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5 Immunological causes of bronchiolitis obliterans after lung transplantation

This chapter will review the factors that may cause bronchiolitis obliterans in human lung transplants, based on the findings in rat lung transplants as presented in this thesis. Furthermore, some strategies wil be proposed that might reduce the development of bronchiolitis obliterans after lung transplantation.

During the last decade the results of lung transplantation have gradually improved as a result of better surgical, postoperative and immunosuppressive treatment. In the same period, however, the results of long-term survival have not changed (1, 2, 3, 4, 5). Soon after the introduction of heart-lung transplantation a considerable number of patients became disabled by severe airflow obstruction in the transplanted lungs. Histological studies on autopsy material revealed that these patients had bronchiolitis obliterans (BO) (7, 8). Unfortunately, the etiology of BO in lung transplants is still unclear and at this moment some pessimists suggest that eventually almost all heart-lung and lung transplant patients will be affected by BO because no effective therapy is available (9). To shed more light on this unsatisfactory situation we investigated in this thesis some possible causes of BO in lung transplants.

Etiologic factors of bronchiolitis obliterans after lung transplantation

Inflammatory responses in the airways play a key role in the development of BO in the lungs of nontransplant patients and in patients with transplanted lungs (10, 11). After lung transplantation a variety of triggers inducing inflammatory responses in the airways act on the lung at the same time, making it difficult to distinguish which is the most important cause of bronchiolitis obliterans in lung transplants. In this chapter we will review three frequently occuring events in lung transplants that may trigger an inflammatory response in the airways of transplanted lung: ischemia, rejection and infection.

Ischemia of the lung transplant

Ischemia of the transplanted lung causes an inflammatory response that may contribute to the development of BO. Early ischemia of the transplanted lung is inevitable during removal from the donor, transport and implantation in the recipient and is the prime cause of the reimplantation response, which to some degree is found in all lung transplants (12, 13). Late ischemia, on the other hand, is possibly related to the absent bronchial circulation in transplanted lungs (14,15).

Early ischemia - the reimplantation response

The reimplantation response is usually mentioned with regard to the impaired lung function early after lung transplantation (11, 16, 17). The reimplantation response is still poorly understood, but it appears to be related with the ischemia of the lung . Ischemia causes damage of cells which becomes apparent after reperfusion of the lung transplant. During the transplantation procedure and during reperfusion of the lung epithelial cells may be damaged, being a trigger for the development of BO.

The damage caused by reperfusion of the ischemic lung transplant is mediated by a non-specific inflammatory response (18, 19). It is generally recognized that leucocytes, especially PMNs, play a central role in the reperfusion injury (20, 21). Upon lung reperfusion PMNs are captured in the microvasculature of the lung. The release of a variety of toxic products, including oxygen free radicals, from the activated PMNs in this inflammatory response causes damage of the lung endothelial cells, lung parenchyma and possibly the airways. Recent experiments in our laboratory demonstrated that after ischemia and reperfusion of rat lung transplants oxygen free radical producing PMNs infiltrate around the airways and in the alveolar septa (Erasmus, personal communication). Similar effects are observed in cardiopulmonary bypass in open-heart surgery after release of the aortic crossclamp and subsequent reperfusion of the lungs (22, 23). Although the function of the lung usually recovers quickly from the reimplantation response, it has previously

been shown that long-term surviving lung isografts can develop fibrotic tissue in the hilar region and the large airways (11). This seems to be related with the period of ischemia since in other studies with significant shorter ischemia times (an average of 30 minutes against more than 60 minutes in the first mentioned study) we could not find such fibrotic changes in the hilar region and large airways of long-term surviving rat lung isografts (24, 25). Similarly, few clinical studies reported that a prolonged ischemia time of the transplanted lungs correlates with the an increased risk for the development of bronchiolitis obliterans (26, 27). These findings indicate that already in the early post-operative phase after lung transplantation an inflammatory response is induced, depending on the duration of the ischemic period of the transplanted lung, that may initiate or at least facilitate the development of BO in the lung transplant.

Late ischemia

Next to the immediate epithelial damage caused by graft ischemia and reperfusion, permanent low grade ischemia of the airways after lung transplantation may exist . The bronchial circulation is not reconstituted during lung transplantation and blood flow to the airways becomes fully dependent on the pulmonary circulation. This might impede oxygenation of the airways by insufficient collateral circulation to the bronchial circulation, which makes the airways vulnerable to ischemia. In well-functioning lung transplants a sufficient blood flow will perfuse the airways, but it has recently been shown that rejection and infection reduce perfusion of the airway epithelium, thereby superimposing ischemic epithelial injury on top of the injury caused by rejection and infection (28, 29). It seems logical that the addition of ischemia of the airways will cause a stronger inflammatory response which eventually results in BO.

Prevention of early and late ischemia

Early ischemia Adequate preservation of the ischemic lung may reduce ischemic damage and the reperfusion injury after restoration of the blood circulation. Although, many centers are putting great efforts in the development of better preservation solutions, ischemia times of the lung are still not extended beyond 7 hours and the reimplantation response still occurs in all transplanted lungs (30, 31).

Another option for treatment is the reduction of PMN activation during reperfusion. In a similar way, the prophylactic use of corticosteroids before cardiopulmonary bypass in open heart surgery effectively reduces the inflammatory response induced by activated PMNs (32). Such effects of corticosteroids have also been demonstrated in experimental ischemic lung injury in dogs (33). Alternatively, protease inhibitors have been demonstrated to reduce the damaging effect of activated PMNs after pulmonary ischemia in dogs (34). Since leucocytes play a crucial role in initiating the inflammatory response caused by reperfusion of the lungs, temporary depletion of leukocytes might eliminate most of their damaging effects. It has been demonstrated in animal experiments that leucocyte depletion by means of leucocyte filtration during cardiopulmonary bypass and lung transplantation significantly reduced the production of oxygen free radicals, particularly during the lung reperfusion period (35, 36).

Late ischemia Restoration of the bronchial circulation would potentially prevent late ischemia of the airways of the transplanted lungs and in this way prevent the occurrence of BO. Restoration of the bronchial circulation could maintain in particular during acute rejection episodes and infections a sufficient mucosal bloodflow (28, 29). Especially the Harefield group strongly advocates reimplantation of the bronchial arteries for the prevention of bronchiolitis obliterans (6, 37). To support their hypothesis the Harefield group is currently performing a prospective study in which in one group of patients the bronchial circulation is restored during the transplantation procedure and in another group a conventional

transplantation is performed (Yacoub, personal communication). The Harefield group recently showed that the airways of double lung transplants were better perfused when the bronchial arteries were reanastomosed, but the data in this study do not support a beneficial effect of bronchial artery restoration in preventing BO, possibly because of the short duration of the study (37). Similarly, the Mayo Clinic group recently showed that routine bronchial artery revascularization is also feasable for single lung transplants and that excellent bronchial artery perfusion can be achieved (38, 39). Although both groups admit that a long-term benificial effect on the occurence of BO has yet to be proven, they speculate that bronchial artery revascularization in lung transplants may favorably affect the occurence of BO.

Rejection

Rejection of the transplanted lung induces a sequence of immunological and inflammatory responses that are most likely contributing to the development of bronchiolitis obliterans. Rejection is a reaction of the recipients' immune system against the non-self antigens of the transplanted lung. Rejection episodes of the transplanted lung are common after lung transplantation, with rates ranging from 50 to 90% in different series over the last 5 years (4, 6, 8, 40, 41, 42). Rejection can be divided into acute rejection and chronic rejection. Although acute rejection is most frequently seen during the first three months after transplantation, it may arise at any time thereafter. Chronic rejection is usually seen in the period of more than 6 months after transplantation.

Acute rejection

In a large number of experimental studies the mechanism of acute pulmonary rejection is relatively well investigated (43, 44, 45, 46). The characteristic features of acute lung allograft rejection are injury of the airway epithelium and blood vessels in the transplanted lung, initially reversible, but eventually becoming irreversible and leading to extensive fibrosis and loss of the lung transplant.

That acute rejection affects the airways has been clearly shown in earlier studies from our department. Prop concluded in his thesis that acute rejection affects the lung transplant more vigorously than other organ transplants in the rat (43, 44, 47, 48). This has been attributed to the bronchus-associated lymphoid tissue of the lung: the BALT contains highly immunogenic lymphoid and non-lymphoid cells (49, 50). Untreated acute lung allograft rejection starts with infiltration of recipient lymphocytes into the BALT (43). Here, recipient helper T lymphocytes interact with donor lymphocytes and antigen-presenting cells in the BALT (dendritic cells and macrophages). These sensitized and activated recipient T helper cells start to produce cytokines which further attract and activate recipient cytotoxic T lymphocytes. Romaniuk showed that from this point the airway epithelium becomes involved in the rejection process: the epithelium starts to express MHC class II antigens and high numbers of lymphocytes infiltrate into the epithelium and the airways (51). The cytokines produced by the activated lymphocytes attract more lymphocytes and simultaneously initiate a non-specific inflammatory reaction with the influx of PMNs and macrophages. In the studies of Prop and Tazelaar it was found that all epithelial cells are destroyed and that the airways are filled with a mixture of damaged cells, fibrin and mononuclear cells, showing the histological picture of bronchiolitis obliterans (45). The airway damage, however, occured only in severe end-stage acute rejection with simultaneous damage of the lung parenchyma which is different from bronchiolitis obliterans after human lung transplantation, affecting only the (small) airways.

Human lung transplantation

Translating the process of acute lung rejection in rats to the clinical situation, however, is not immediately possible. Some important differences between the above mentioned animal studies and the clinical situation most be mentioned. First, in animal studies on acute lung rejection, the lung tranplant recipients are usually not immunosuppressed, to allow full rejection of the lung allograft. This is in sharp contrast with human lung transplantation, where agressive

immunosuppression is maintained. As a result acute rejection episodes in humans are usually low-grade and do not advance to an end-stage with complete necrosis of the airways (6, 8, 10, 52, 53, 54). Second, the role of BALT in human lung allograft rejection is disputed since BALT is not constituent part of the human lung (55, 56). Nevertheless, a link between acute rejection episodes and bronchiolitis obliterans was suggested in recent studies from Pittsburgh and Papworth (57, 58, 59). They found a possitive correlation between the number of acute rejection episodes and the occurence of BO. Also in these studies the grade of acute rejection was much milder than the severe acute rejection in rats. The authors conclude, however, that repeated insults of low-grade acute rejection or ongoing low-grade rejection, will cause a chronic continuous inflammatory response in the airways which will ultimately lead to bronchiolitis obliterans.

Chronic rejection

In contrast to acute rejection, chronic lung allograft rejection is not clearly defined, let alone understood. Pathologically, chronic lung rejection is defined as fibro-obliterative lesions involving vessels and airways (52, 60). However, since most lung transplant recipients ultimaly suffer from progressive airflow limitation, most emphasize is put on the airway changes, i.e. bronchiolitis obliterans. For that reason, bronchiolitis obliterans is regarded by many authors to be the equivalent of chronic lung allograft rejection (53, 54, 57, 58, 59). The idea that chronic rejection is the sole trigger of BO is supported by data from the Pittsburgh group showing an increased alloreactivity in cells lavaged from lung transplant patients with BO (60, 61). Further supportive evidence was found in a few immunohistological studies showing enhanced expression of MHC class II antigens on the bronchiolar epithelium of patients with bronchiolitis obliterans (62, 63) together with infiltration of cytotoxic T lymphocytes (64). Bronchiolitis obliterans, however, is the end-stage of an inflammatory process (9), and is usually diagnosed in this late stage. As a consequence little is known about the evolution of the

presumed chronic rejection process to BO in patients. Another complicating factor is that information from clinical studies is clouded by a large number of interfering factors. Animal models under standardized and controlled conditions are therefore required to investigate the process of chronic lung allograft rejection. Until recently, however, no reproducible experimental models were available to investigate chronic lung allograft rejection, in contrast to the abundant availability of experimental models for acute rejection. This situation is not unique for lung transplants since a similar lack of chronic rejection models limits the understanding of chronic rejection in other organ transplants (66).

With our animal model of chronic rejection we confirmed that isolated chronic rejection can cause airway changes in long-term surviving rat lung allografts (67, 68). The immunological process associated with the airway changes was characterized by MHC class II expression on the bronchial epithelium, accumulation of dendritic cells in the submucosa and lymphocytes infiltrating in the submucosal tissue (67, 69). Although these changes remained focal and mild it indicated that a local inflammatory response damaged the bronchial epithelium and might serve as a trigger for the development of bronchiolitis obliterans.

From our findings it is clear that chronic rejection induces milder airway damage in rat lung allografts than untreated acute rejection does. This seems to be related with differences in the inflammatory response associated with either acute or chronic rejection. The inflammatory response during acute rejection is more intense and shows a predominance of cytoxic T cells and activated, inflammatory macrophages (46, 70), while in chronic rejection helper T cells and non-activated macrophages are most common (67, 68, 71). Also, in the clinical study of chapter 4.2 we found a more severe inflammatory response during acute lung allograft rejection than during chronic rejection (72). Similary, clinical and experimental studies in heart, liver and kidney allografts found a more intense inflammatory response during acute

rejection than during chronic rejection (73, 74, 75, 76). Also in these studies acute rejection was associated with more severe tissue damage than chronic rejection.

From our experimental study we conclude that isolated chronic rejection indeed is clearly one of the causes of BO in lung transplants, but not the only one. In our view BO is rather the end-stage of a multifactorial process and should not be considered as the equivalent of chronic rejection.

Prevention and treatment of rejection

Tissue matching

Since lung allograft rejection is initiated by incompatibilities of tissue antigens, especially the major histocompatibility (MHC) antigens, between the donor and recipient, matching of donor and recipient tissue antigens would reduce rejection and reduce the incidence of BO. This positive impact of matching has been demonstrated in retrospective studies in lung tranplants (4, 5) Similarly, the possitive effect of matching donor and recipient has been clearly been shown after kidney transplantation where matching results in better long-term graft survival (77). In lung transplantation, however, prospective matching of donor and recipient is difficult to achieve, because the short preservation time of the explanted lung does not allow sufficient time to perform the tissue-typing and subsequent transport to the best matching patient. A second major obstacle is the low number of available donor lungs and the limited period of time that patients can wait for a transplant. So, even when prospective matching of donor and recipient would be technically feasible, the chance of a particular patient to get a lung offered with matching HLA-combinations would be very small.

Rejection detection

Since adequate matching of donor and recipient remains difficult, early diagnosis and treatment of rejection is essential. Clinical symptoms, although widely used, are not suitable for early detection of acute and chronic rejection since they appear relatively late in the rejection process (6, 7, 8, 59), therefore other tests are required for early detection of rejection. Up to now no absolutely satisfying technique for

rejection detection exists. Lung function tests, chest X-rays, bronchoalveolar lavage and transbronchial biopsies (TBBs) are all used after lung transplantation (6). TBBs are still considered to be the gold standard in the diagnosis of lung rejection. Although the role of TBBs is well defined (52, 79, 80), high sensitivity and specificity can be only be obtained in few experienced centers (79, 80, 81). Moreover, the quantity of the lung tissue obtained by TBBs is often very limited because in most TBBs only blood vessels and alveolar septa are available for analysis. Therefore, early diagnosis of rejection on histopathological grounds is often difficult using TBBs. We showed in the collaborative study with Papworth Hospital described in chapter 4.2 that the sensitivity of TBBs can be improved by the use of phenotype analysis of the infiltrating cells in the biopsy tissue (72). This phenotype analysis might be helpful in early diagnosis of rejection and the timely start of treatment, thereby limiting the damage inflicted on the transplanted lung.

Although reliable, the procedure of obtaining TBBs is an invasive method, thereby introducing the risk of infectious and bleeding complications. Non-invasive rejection detection methods that could detect early immunological changes in the lung transplant would resolve this problem. Cytoimmunomonitoring (CIM) of lymphocytes in the peripheral blood seems to have some value in diagnosing acute rejection episodes, but is not sensitive in diagnosing chronic rejection (82, 83). Another non-invasive detection method could be the evolution of PET-scanning in combination with immunological markers. This method has the potential to become a sensitive lung allograft rejection detection method, possibly eliminating the need for invasive methods.

Immuno-suppression

Immunosuppression for prevention and treatment of rejection is still *a conditio sine qua non* in human lung transplantation. Cyclosporine A (CsA), with its selective and reversible inhibition of immunocompetent T-lymphocytes, is considered today as the first-line treatment to suppress lung allograft rejection. Although CsA improved the results of

organs transplantation tremendously, it is still not the perfect immunosuppressive drug. Systemic CsA administration has severe side-effects, like nephrotoxicity and hypertension, and is also not able to prevent faillure of organ transplants in the long-term. Induction of local immunosuppression directly in the lung transplant may be an alternative approach to achieve better control of rejection and avoid systemic side-effects during immunosuppressive therapy. Both the induction of the immune response by the MHC-complex antigens and the effector mechanisms during the cellular immune response are largely regulated locally in the lung transplant (43, 52, 72) and local application of immunosuppressive agents will therefore directly block induction and effectuation of immune and inflammatory responses. The feasibility of direct delivery of CsA in rat lung transplants has recently been demonstrated by the Pittsburgh group (84, 85). They showed that aerosolized CsA can prevent rejection of allografted rat lungs effectively. They also showed in a preliminary study in patients that local application of an other powerful immunosuppressive drug, FK506, can reverse lung rejection that could not be treated with CsA and steroids (Duncan, personal communication). Similarly, it has been shown in experimental kidney and heart transplants that local application of CsA is effective as anti-rejection treatment (86, 87). These data indicate that local application of immunosuppressive drugs (CsA, FK506, steroids) will suppress rejection, particularly in the airways of lung transplants. In this view it would be the ideal prevention of rejection as a trigger of BO.

Infections

Infections, being a prime cause of epithelial damage, can cause BO in normal individuals. Since infections are a major complication after lung transplantation (88-96), they may be of even more importance as a cause of BO in transplanted patients. In non-transplant patients post-viral BO is mostly seen in young children and immunocompromised older patients (9, 97, 98, 99, 100, 101). A characteristic feature of these patients is a reduced pulmonary defense, in particular the

inadequate production of antibodies, especially IgA, in the lung (9, 101, 102, 103). In parallel, we hypothesized that the high number of intrapulmonary infections in lung transplant recipients is likely to result from an impaired lung defense in these patients. In this thesis we provide evidence that the lung defense is impaired after lung transplantation: not only the non-specific, or mechanical, defense mechanisms, as was described earlier (104, 105), but in particular the antigen-specific immune responses.

Non-specific defense

In normal lungs, the mechanical part of the lung defense system is essential to protect the airway epithelium against contamination with potential pathogens, thereby preventing subsequent infection and airway damage. It should be realized that the lung is prone to be contaminated during the transplantation procedure. First, most lung donors are intubated, and intubation often causes contamination of the airways, which is transmitted to the transplanted lung. And second, there is a high risk of contamination at the time of surgery since the trachea and bronchi are transected, providing an easy porte d'entrée of microorganisms, either from the recipient or donor side. Immediately after lung transplantation several components of the mechanical defense system are impaired. Ischemia may damage the donor lung before, during or after harvesting and preservation (as described above). It has been shown that ischemic injury to airways reduces their capability to clear micro-organisms from the airways (106). In addition, it has recently been reported that interruption of the bronchial artery circulation contributes to increased severity of pneumonia in rats, with or without lung transplantation (107). Furthermore, it has been shown that severence of nerves after lung transplantation impairs the function of the mucociliar escalator thereby eliminating an important clearance mechanism in the lung (104, 108). Finally, injury to the phrenic nerve may cause poor diaphragmatic function and lead to impaired coughing and atelectasis of the transplanted lung.

Specific defense

In this thesis we have demonstrated that also the antigen-specific immune reactions against intrapulmonary antigens in the transplanted lung are impaired after lung transplantation. Both systemic antibody responses and local antibody responses in the transplanted lung in allogeneically transplanted rats appeared to be impaired and contributed to a reduced pulmonary defense in these lungs.

Systemic antibody response

Systemic responses against antigens in lung allografts were low, but their relevance for development of BO is as yet uncertain. Lymphatics appeared to be crucial for a normal systemic antibody response against intrapulmonary antigens in the lung. We found that the antibody responses against particulate antigens were impaired in the early post-operative period following lung transplantation as long as the lymphatics were interrupted (109, 110, 111). Apparently, the systemic immune defense against these intrapulmonary antigens depends on an adequate response in the draining paratracheal lymph nodes. This weak systemic antibody response may in part explain the high incidence of infections early after clinical lung transplantation (89-95).

It was surprising and not well understood that also the systemic antibody response against a respiratory viral infection in allogeneically transplanted rats was decreased. A normal response would be expected as the infection was cleared normally in the contra-lateral, nontransplanted lung (112, 113). Whatever the mechanism of the reduced response is, the consequence is that lung allotransplants are susceptible to pulmonary infections. These infections will cause inflammatory reactions in the airways which contribute to BO, as we have demonstrated in chapter 2.4.

Local antibody response in lung transplants

The local antibody response against a respiratory viral infection was abnormal in rat lung allografts. We found that a viral infection did not induce an antibody response in the BALT of the allogeneic lung transplants (112, 113). This impaired local antibody response resulted in prolonged presence of the virus in the epithelium of the airways. This protracted presence of the virus resulted in a severe inflammatory response and subsequent airway damage, eventually causing the histopathological picture of BO. In lung transplants this sequence of events has not been demonstrated before, but in patients with impaired local IgA production a similar susceptibility to infections and subsequent BO has been observed (101, 102).

The inadequate antibody response in the BALT after viral infection in lung allografts could be attributed to damage of the BALT in these lungs. The BALT in long-term surviving rat lung allografts becomes fibrotic and there is no functional uptake of particulate antigens and migration of lymphocytes we have show in chapter 3.5 (114, 115). Damage of the BALT is most likely caused by rejection. Prop showed that the BALT is a target of lung allograft rejection (43, 47, 48), which destroys the donor lymphocytes in the BALT. Also, after cyclosporine treatment rejection damages the BALT, even when the lungs survive indefinately (114, 115, 116). In similar experiments the Peyers' patches were found to become fibrotic in long-term surviving rat small bowel transplants with chronic rejection (117).

Our data convincingly shows that the BALT is destroyed in transplanted lungs resulting in viral infections causing a delayed clearance of the virus and a severe and prolonged inflammatory reaction. This leads eventually to BO in these lungs.

The BALT in human lung transplantation

We realize that our concept of the role of BALT in the development of BO in lung transplants is mainly based on experimental lung transplantation in rats. Extrapolation to human lung transplantation is therefore not immediately

possible. In humans, the situation is different in many respects and one could argue whether there is any effect at all of the local immune system transplanted within the lung. Despite the differences between rat and man, we think that our concept also applies to clinical transplantation.

BALT is not a constituent part of human lungs (50, 55) as it is of rat lungs. With a careful selection of donors for lung transplantation, it is likely that they are free from pulmonary infections, and therefore have no BALT-like lymphoid structures in the lungs. Nevertheless, the human lung also contains an extensive lymphoid system, although it is not structured in dense aggregates like BALT in rats. Besides the pulmonary parenchyma, also lymphnodes transplanted with the lung contain lymphocytes. It has been estimated that with a single lung between 2 and 5x10.10 mononuclear cells are transmitted in total. This is clearly a large amount of cells that may a similar role after lung transplantation as BALT.

In addition, it is hard to investigate whether the infection incidence after clinical lung transplantation increases as a result of a damage of the local defense system in the lung. For, all patients receive immunosuppressive agents that make them more susceptible for infection. Comparing lung transplant recipients with recipients of other organ grafts, however, shows that they many infection episodes: for lung transplant recipients 3.36 episodes per patient in the first postoperative year compared to 1.41 and 1.83 episodes for heart and liver recipients, respectively. Bases on this comparison it is not unlikely that the defense in the transplanted lungs is impaired as it is in rats. At least there is evidence that the number of peribronchial plasma cells as a possible equivalent of BALT is reduced in human lung transplants with chronic rejection (118).

Prevention and treatment of infections

Lymphatics

Since transplantation means removal of a donor organ from one body and reconnecting it to another body, severence of blood vessels, lymph vessels and nerves cannot be avoided. Theoretically reconstruction of these hilar structures would result in normal non-specific lung defense. However, reconstruction of lymph vessels and nerves is difficult, if not impossible, and is not attempted in clinical lung transplantation. An important finding from our studies is that lymphatics from the lung are only temporarily interrupted, and will, even in allogeneically transplanted lungs, regenerate rather quickly. Since this *natural* reconstruction takes place within two weeks after transplantation it is particularly important to reduce the possible sources of infection in this early post-transplantation period. The most important sources of infection in this period are the diagnostic interventions, e.g. intubation, bronchoalveolar lavage and transbronchial biopsies. Our findings emphasize the importance of careful antibiotic measures during this period.

The BALT

Since rejection is probably the most important cause of the damage of the BALT, improved immunosuppression might preserve the BALT and thus the local immune defense against infection in the transplanted lung. Prop previously showed that irradiation of the donor lung delayed untreated rejection (43, 47, 48). By irradiation most immunocompetent cells in the lung and particularly in the BALT, are destroyed and the response from the host is less intense (47, 48). In combination with immunosuppression this might prevent damage of the BALT. The empty, but intact structure of the BALT might be repopulated with recipient immune cells and function as a normal BALT. Experimental lung transplantation could serve as a model for testing this hypothesis.

A second therapeutic strategy could be to use the so-called common mucosal immune system as a source of protective immunoglobulins. It has been shown by several studies that lymphoblasts, especially precursors of IgA producing cells deriving from the gut immune system, do not only home

preferentially to the submucosa of the gut, but also to the respiratory tract (119, 120, 121). By using this system lung transplant patients can be vaccinized via the oral route against potential dangerous microorganisms in the lung. This has already succesfully been demonstrated in non-transplant patients with viral (122) and bacterial antigens (123).

Another option might be the local application of immunoglobulins, especially IgA, directly into the transplanted lung. These immunoglobulines could be administered by aerosols to achieve direct protection of the airway epithelium, thereby preventing pathogens adhering to its surface. At present, however, no clinical or experimental data is available to demonstrate the feasibility of this type of preventive therapy.

Inflammation: the central pathway to bronchiolitis obliterans after lung transplantation

In the above paragraphs three potential causes of bronchiolitis obliterans in lung transplants have been described: ischemia, rejection and infection. They have been described as isolated mechanisms, but in clinical lung transplantation they will always act in combination, conceivably mutually potentiating their isolated contributions. Although the induction of the inflammatory response by ischemia, rejection and infection may be different, once induced the response can be considered to follow a common inflammatory pathway causing the actual tissue damage. We showed that the simultaneous occurence of chronic rejection and viral infection caused an inflammatory response which was more intense than after their isolated occurence, and that it eventually caused bronchiolitis obliterans.

The mechanisms by which these factors mutually potentiate each other are still not clear. The ischemia of the lung and infections might activate the rejection process by activation of lymphocytes and upregulation of class II MHC antigens on the bronchial epithelium. Such increased alloreactivity has been demonstrated clinically in cardiac transplant recipients after active influenza vaccination (123)

and in lung transplant recipients after CMV infection (55, 124) and pneumocystis infection (89). Another option is the impairment of the immunological defense mechanisms of the lung that cause severe infections. These infections can subsequently cause BO in lung transplants by similar mechanisms as in nontransplant patients. Most importantly, inflammation plays the pivotal role in the actual damage of the airways after lung transplantation, irrespective of the initial triggers, or combination of triggers. Therefore, it would be a logical approach to suppress the inflammatory response in any clinical event, irrespective whether it is ischemia, rejection or infection. This is supported by the clinical observation that even without exact diagnosis, a deteriorating lung function in lung transplant recipients can be favorably improved by treatment with corticosteroids (10, 17, 125, 126), even in patients that later turned out to be infected (126). A recent preliminary clinical study suggested that the oxygen free radical scavenger allopurinol might reduce the development of bronchiolitis obliterans in lung transplant patients with declining lung function (127). Similarly, it is since long (128) recognized that in other lung diseases, like asthma, non-specific inflammation in the lung plays a key role in the development of histopathological changes. Inhibition of the inflammatory response is now an important component in the treatment of asthma patients (129, 130). Apperently, the lung reacts with a strong inflammatory response on whatever foreign invader under abnormal circumstances, like asthma and lung transplantation.

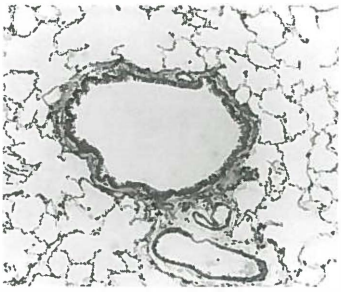
Anti-inflammatory treatment with low dose systemic corticosteroids is a standard component of the immunosuppressive protocols of most transplant centers. It is conceivable, however, that in case of a severe inflammatory reaction, the levels of corticosteroids in the lung are too low to suppress this inflammatory reaction adequately. Local application of corticosteroids directly into the lung, like in patients with asthma, might be an effective treatment to break the vicious inflammatory circle.

Conclusion

By using an immunologically well defined model for lung transplantation we were able to unravel different causes that contribute to bronchiolitis obliterans in lung transplants. We showed that chronic rejection causes mild airway damage in lung allografts. In addition, chronic rejection causes structural changes in the BALT, resulting in an inadequate function of the local defense in the transplanted lung. This makes the lung very susceptible to infection, resulting in a vicious circle of inflammatory responses which will eventually lead to BO (see adjoining page). We speculate that this vicious circle may be broken by agressive anti-inflammatory treatment thereby preventing the development of BO in lung transplants.

The "Groningen" model for the development of bronchiolitis obliterans after lung transplanation

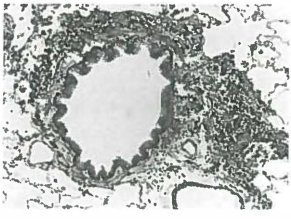
Lung transplantation



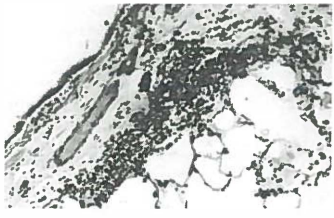
Normal bronchiole

Normal BALT

Chronic rejection

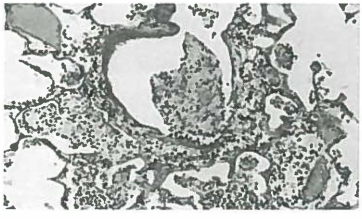


lymphocytic infiltration
around the bronchiole



Destroyed BALT

Infection



Bronchiolitis obliterans

5.1 Bronchiolitis obliterans after lung transplantation

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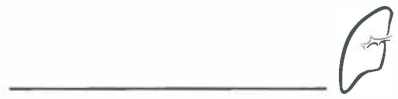

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5.2 Summary

1. The progress of lung transplantation

From a review of the current literature it can be concluded that during the last decade the results of lung transplantation have progressively improved as a result of better patient selection and surgical, postoperative and immunosuppressive treatment. Nowadays, after lung transplantation many recipients are able to live a relatively normal life. In the same period, however, the long-term survival has not improved noticeably. In approximately 50% of the lung transplant recipients a clinical syndrome of progressive respiratory dysfunction develops. It is even predicted that eventually all transplanted lungs will be affected by this syndrome. This progressive lung dysfunction correlates with obstruction and destruction of the small airways in the transplanted lung, known as bronchiolitis obliterans (BO). Unfortunately the etiology of BO in lung transplants is still unclear, and as a consequence no effective therapy is available. In non-transplant patients BO is the result of a tissue reaction upon damage of the respiratory epithelium, irrespective of the initial trigger that caused the epithelial damage. In lung transplanted patients, various factors have been mentioned that can cause the epithelial damage thereby triggering the development of BO. Among these factors chronic rejection and viral infection are most suspected. Especially chronic rejection is considered by many investigators to be the prime cause of BO and is often regarded as the equivalent of BO. However, in the clinical situation a large number of interfering factors makes it difficult to analyse the causes and mechanisms that lead to BO.

In this thesis we investigate the contribution of chronic rejection and viral infection in the development of airway damage after lung transplantation in a rat model under standardised conditions. Furthermore, we analyse the immune and inflammatory responses associated with chronic rejection and viral infection as mechanisms leading to BO in these lung transplants.

2. Bronchiolitis obliterans in rat lung transplants

In the studies described in chapter 2 it appeared that airway damage similar to BO appeared to be caused only by the combination of chronic rejection and viral infections in long-term surviving allogeneic lung transplants. With our animal model of chronic rejection we confirmed that isolated chronic rejection indeed can cause airway changes in long-term surviving rat lung allografts (chapter 2.2). The immunological process associated with the

airway changes was characterized by MHC class II expression on the bronchial epithelium, accumulation of dendritic cells in the submucosa and lymphocytes infiltrating in the submucosal tissue. These findings indicate that a local immune response associated with chronic rejection is able to damage the bronchial epithelium and then serves as a trigger for the development of bronchiolitis obliterans. The airway changes in these rat lung allografts, however, were mild and only present in the large airways. This is different from bronchiolitis obliterans after human transplantation where damage is severe and predominantly located in the small airways. Nevertheless, these experimental findings proved that chronic rejection can cause airway damage.

In the next series of experiments we investigated whether a respiratory viral infection could induce bronchiolitis obliterans-like airway damage in long-term surviving rat lung transplants in the absence or presence of chronic rejection (chapter 2.3). We found that the respiratory viral infection induced severe damage in the airways of the lung transplants, but only in combination with chronic rejection. In these infected lung transplants the airway damage in the bronchioles of the allogeneic lung transplants showed the typical features of bronchiolitis obliterans: epithelial damage, formation of granulation and scar tissue in the submucosa and subssequent obliteration of the airway lumen, very much like bronchiolitis obliterans in lung transplant patients. In the absence of chronic rejection in the syngeneically transplanted and nontransplanted lungs, changes in the airways were only transient and returned to normal in 8 weeks after infection, without persisting scar tissue. These findings clearly indicate that viral infections together with chronic rejection play a crucial role in the development of BO in lung transplants.

The mechanism leading to the severe airway damage in infected allogeneic lung transplants was found to be characterized by an intense inflammatory response (chapter 2.4). The viral infection aggravated the chronic inflammatory response in the airways of the noninfected allografts which was caused by chronic rejection. The active inflammatory response in the infected allografts was characterized by inflammatory macrophages and O_2^--producing PMNs together with a strong expression of MHC class II antigens on the bronchial epithelium. Surprisingly, the immune response after infection, characterized by the infiltration of CD4 and CD8-positive T cells and B cells, was highly abnormal in the allografts: in particular the infiltration of CD8-positive cells started slowly, but then persisted till the end

of the observation period, while B cell responses were not initiated at all. Our findings indicate that after viral infection in rat lung allografts severe airway damage is induced by severe inflammation. This may be the result of an inadequate antiviral defense.

3. Immune responses in infected rat lung transplants

Systemic immune responses and local immune responses in the lung are essential for an adequate defense against intrapulmonary antigens. Especially the local antibody production in the BALT of the lung is suggested to be essential a first-defense against intrapulmonary pathogens, as mentioned in the introduction (chapter 3.1). The absence of a clear histological immune response after viral infection in the allogeneic lung transplants (chapter 2.4) raised the question as whether the antigen-specific immune response against respiratory viral infections is impaired in transplanted lungs. Therefore we investigated whether systemic and local immune responses against intrapulmonary antigens, sheep red blood cells (SRBC) and Sendai virus, are impaired in allogeneic lung transplants.

First, the systemic antibody response against intrapulmonary administered SRBC appeared to be impaired, however only in the early postoperative period (chapter 3.2). At one month after transplantation a normal systemic antibody response against SRBC could be mounted in the allogeneic lung transplants. This temporary impairment could be attributed to the interruption of the pulmonary lymphatics by the transplantation procedure and their subsequent regeneration (chapter 3.3). Interruption of hilar lymphatics by hilar stripping prevented transport of antigens to the hilar lymphnodes and subsequent induction of an antibody response. After regeneration of the lymphatics two weeks after operation, antigens were transported to the lymphnodes again and an antibody response can gradually recover to normal values. Since this *natural* reconstruction of lymphatics takes place within two weeks after transplantation it is particularly important to reduce the possible sources of infection in this early post-transplantation period. The most important sources of infection in this period are the diagnostic interventions, e.g. intubation, bronchoalveolar lavage and transbronchial biopsies. Our findings emphasize the importance of careful antibiotic measures during this period.

Second, also antibody responses against Sendai virus, both the local and systemic response, were found to be absent upon respiratory infection in

the allogeneic lung transplants (chapter 3.4). Most strikingly, the number of antibody-forming cells in the local defense system in the lung, the BALT, did not increase after infection. As an apparent consequence the virus was longer present in these lungs. These results indicate that the BALT of the allografted lung was unable to respond properly against the respiratory infection.

The findings from chapter 3.4 made us hypothesize that the BALT was somehow damaged by the allotransplantation that made it unable to function adequately after infection. Therefore, we investigated 3 prerequisites for a normal function of the BALT, i.e. structure, uptake of antigens and lymphocyte migration to the BALT in long-term surviving lung allografts (chapter 3.5). We found that in the allografts the BALT was defective in all investigated aspects. It was reduced in size and lymphocyte density and was largely replaced by fibrous tissue. The fibrosis in the BALT hampered the uptake of particles form the airways and lymphocyte migration to the BALT. These damage of the BALT is most likely caused by rejection, as the BALT in syngeneic lung transplants was perfectly normal.

Extrapolation to human lung transplantation, however, is not immediately possible. In humans the BALT is not a similar constituent part of the lung as it is of rat lungs. Nevertheless, the human lung also contains an extensive lymphoid system although not always structured in dense aggregates like BALT. Furthermore, it is hard to investigate whether infection incidence after clinical lung transplantation increases as a result of a damage of the local defense system. All patients receive immunosuppressive agents that makes them more susceptible for infection. Comparing lung transplant recipients with recipients of other organ grafts, however, shows that they suffer from much more infection episodes than heart and liver recipients. Nevertheless, it is not unlikely that the defense in the transplanted lungs in humans is impaired by the same mechanisms as it is in rats.

Our findings in chapter 3 demonstrated that primarely the local immune defense against intrapulmonary antigens is permanently impaired in allogeneic lung transplants. As a consequence of this absent local antibody response intrapulmonary pathogens will be longer present in the transplanted lung, thereby maintaining an inflammatory response against the virus which will eventually cause severe damage and might lead to BO.

4. Immune responses in human lung transplants

In patients, we presume that BO is caused by similar mechanisms of derailed inflammatory and immune responses in lung transplants as we have found in rats. Early detection of these responses in the lung transplant would enable timely treatment and consequently prevention of development of BO. Such an analysis of responses in the transplanted lung can be made most reliably by examination of pulmonary tissue obtained by biopsy (chapter 4.1). Transbronchial biopsies have been advocated as an effective diagnostic procedure for the detection of lung allograft rejection and infection. However, TBBs often provide insufficient tissue to permit fullfillment of strict histologic criteria needed to diagnose allograft rejection or infection.

In a clinical study we found that immunohistochemical phenotyping of infiltrating cells during lung rejection might be helpful for early detection of rejection-mediated immune and inflammatory responses (chapter 4.2). Even during mild acute and chronic rejection specific immune and inflammatory responses can be found in the perivascular tissue, which corresponds with immune and inflammatory responses in the peribronchiolar tissue. T cells were present both in acute and in chronic rejection, but did not differentiate between them. In contrast, B cells with antibody deposition in the blood vessels were mainly present during early chronic rejection and not during acute rejection. Furthermore, acute rejection was characterized by a more intense inflammatory response than chronic rejection, as demonstrated by the presence of activated macrophages, that were absent during chronic rejection. Our study clearly shows that acute and chronic rejection can be detected by immunohistochemical phenotyping of cells involved in early stages of the immune and inflammatory responses. Therefore, this technique may help to improve the diagnosis and treatment of rejection in lung transplants, thereby preventing the development of fatal BO.

5. The etiology of BO in lung transplantation - Conclusions

In the general discussion of this thesis we conclude that bronchiolitis obliterans in lung transplants is caused by a severe inflammatory response triggered by the combination of rejection and infection (chapter 5.1). Besides that, development of bronchiolitis obliterans may be further triggered by other factors that induce inflammatory responses, such as early and late ischemica of the transplanted lung. Irrespective of the initial

trigger, or combination of triggers, inflammation plays the pivotal role in the induction of airway damage after lung transplantation. In this view, it would be a logical approach to suppress the inflammatory response aggressively in any clinical event, irrespective whether it is ischemia, rejection or infection. Anti-inflammatory treatment with low dose systemic corticosteroids is a standard component of the immunosuppressive protocols of most transplant centers. It is conceivable, however, that in case of a severe inflammatory reaction, the levels of corticosteroids in the lung are too low to suppress this inflammatory reaction adequately. Local application of corticosteroids directly into the lung, like in patients with asthma, might be an effective treatment to break the vicious inflammatory circle.

By using an immunologically well defined model for lung transplantation we were able to unravel different causes that contribute to bronchiolitis obliterans in lung transplants. We showed that chronic rejection causes mild airway damage in lung allografts. In addition, chronic rejection causes structural changes in the BALT, resulting in an inadequate function of the local defense in the transplanted lung. This makes the lung very susceptible to infection, resulting in a vicious circle of inflammatory responses which causes severe airway damage, ultimately leading to bronchiolitis obliterans. We speculate that this vicious circle may be broken by agressive anti-inflammatory treatment thereby preventing the development of BO in lung transplants.

6 Summaries, for those unfamiliar with lung transplantation

6.1 Nederlandse samenvatting

Je lichaam, voor driekwart samengesteld uit water, plus wat aardse mineralen,
een handjevol.
En in je die grote vlam waarvan je de aard niet kent.
En in je longen, altijd en altijd weer opgenomen in de borstkas,
de lucht, die schone vreemdeling zonder wie je niet kunt leven.

MargueriteYourcenar

1. De ontwikkeling van de longtransplantatie

Door de stormachtige ontwikkeling van de longtransplantatie in de laatste tien jaren kan deze ingreep tegenwoordig met groot succes bij patiënten met ernstige longziekten worden uitgevoerd. De meeste ontvangers van longtransplantaten kunnen weer een normaal leven leiden en een groot aantal is zelfs weer aan het werk. Dit lijkt allemaal zeer rooskleurig, maar het blijkt dat ook hier schone schijn bedriegt. De resultaten in het eerste jaar na longtransplantatie zijn de laatste 10 jaar dan wel enorm verbeterd, na dat eerste jaar vermindert bij een groot deel van de patiënten de functie van de getransplanteerde long. Bij veel patiënten loopt ze zelfs zover terug dat deze patiënten eraan overlijden. De in de geneeskunde gebruikte 'vijfjaarsoverleving' om het succes van een behandeling uit te drukken, is na longtransplantatie minder dan 50%, ongeacht het type longtransplantatie. Dit betekent dat 5 jaar na longtransplantatie nog maar de helft van de getransplanteerde mensen in leven is. Het blijkt dat bijna bij alle patiënten die overlijden, de longfunctie afneemt als gevolg van een vernauwing van de kleine luchtwegen van de long door een ontstekingsreactie. Deze aandoening wordt ook wel bronchiolitis obliterans genoemd. De oorzaak van bronchiolitis obliterans na longtransplantatie is helaas niet duidelijk en daardoor slecht te behandelen.

Bronchiolitis obliterans begint met schade aan het epitheel, de bekleding, van de luchtwegen, en dat is bij patiënten na longtransplantatie niet anders dan bij niet-getransplanteerde mensen die bronchiolitis obliterans ontwikkelen. Bij patiënten die niet getransplanteerd zijn kan bronchiolitis obliterans ontstaan na epitheelschade door virale infecties of inademing van toxische gassen. De ontstekingsreactie die na deze epitheelschade in de luchtwegen ontstaat, is in de meeste gevallen gelijk, ook al zijn de oorzaken verschillend. Na de eerste beschadiging van het epitheel ontstaat er een ontstekingsreactie van het lichaam om de schade te beperken en te herstellen. Tijdens deze reactie migreren verschillende soorten cellen vanuit het bloed naar de plaats van de beschadiging.

Macrofagen, neutrofiele granulocyten en in sommige gevallen lymfocyten stromen in grote getale naar de beschadiging. Als het goed is zorgen deze cellen ervoor dat de beschadigde epitheelcellen worden opgeruimd en dat nieuwe epitheelcellen kunnen uitgroeien, tot uiteindelijk de beschadiging geheel verdwenen is. Dit hele proces van opruimen en herstellen noemen we een ontstekingsreactie, die ophoudt als alles hersteld is. In het geval van bronchiolitis obliterans blijft de ontsteking echter te actief en treedt geen echte herstelfase op. Door deze aanhoudende ontsteking wordt uiteindelijk vooral littekenweefsel gevormd en bijna geen epitheel cellen. Dit littekenweefsel is erg stug en zorgt ervoor dat de luchtwegen zich blijvend vernauwen.

Bronchiolitis obliterans een vrij zeldzame ziekte bij patiënten die niet getransplanteerd zijn. Waardoor het komt dat bronchiolitis obliterans na longtransplantatie zo vaak optreedt is niet duidelijk. Op dit moment wordt verondersteld dat de ontsteking in luchtwegen wordt veroorzaakt door een vorm van chronische immuunreactiviteit van de ontvanger tegen de getransplanteerde long, d.w.z. chronische afstoting, en door (virus) infecties van de long, die na transplantatie zeer vaak optreden.

Chronische afstoting wordt op dit moment beschouwd als de belangrijkste oorzaak van bronchiolitis obliterans. In de classificatie van de International Society for Heart and Lung Transplantation is de bronchiolitis obliterans dan ook opgenomen in de groep "chronic airway rejection". Een duidelijke aanwijzing dat bronchiolitis obliterans samenhangt met afstoting blijkt uit twee klinische bevindingen. Het blijkt dat cellen van de ontvanger in de getransplanteerde long kunnen worden aangetroffen die reactief zijn tegen de weefselantigenen van het transplantaat, voordat bronchiolitis obliterans manifest wordt. In de tweede plaats is beschreven dat bronchiolitis obliterans vaker voorkomt in longen die meerdere perioden van acute afstoting hebben doorgemaakt.

De samenhang van infectie met bronchiolitis obliterans wordt voortdurend genoemd, maar het mechanisme ervan is niet duidelijk. Vooral infectie met het Cytomegalovirus (CMV) is aangegeven als belangrijkste oorzaak van bronchiolitis obliterans, maar inmiddels wordt steeds duidelijker dat ook andere (virale) infecties van long en luchtwegen bronchiolitis obliterans kunnen veroorzaken. Tijdens een ogenschijnlijk onschuldige luchtweginfectie wordt gezien dat de functie van het longtransplantaat afneemt en zich daarna niet herstelt. In longbiopten wordt dan eerst een aspecifieke bronchiolitis gezien, die zich vervolgens geleidelijk ontwikkelt tot een duidelijke bronchiolitis obliterans. Hoewel afstoting en infectie dus oorzaken kunnen zijn van bronchiolitis obliterans in longtransplantaten, is het optreden en het verloop ervan bij individuele patiënten

onvoorspelbaar. Het is daarom vaak onduidelijk wat de belangrijkste oorzaak is van bronchiolitis obliterans in de getransplanteerde long.

Door dit gebrek aan inzicht over het ontstaan van bronchiolitis obliterans dreigt er een soort impasse te ontstaan in de ontwikkeling van de longtransplantaties bij mensen. Proefdieronderzoek zou mogelijk inzicht kunnen geven in het ontstaan van bronchiolitis obliterans na longtransplantatie. Het grote voordeel van proefdieronderzoek is, dat de verschillende factoren waarvan wordt verondersteld dat ze bronchiolitis obliterans in de getransplanteerde long veroorzaken, afzonderlijk en in combinatie kunnen worden onderzocht. Bij proefdieren zoals ratten is het mogelijk door middel van inteelt stammen te krijgen waarvan alle individuen de zelfde weefselkenmerken hebben. Als bij transplantatie de donor en ontvanger van dezelfde stam zijn zal er geen afstoting van het getransplanteerde orgaan optreden, doordat hun weefselantigenen hetzelfde zijn: dit noemen we een syngene transplantatie. Als de donor en ontvanger echter van verschillende stammen zijn, de allogene transplantatie, zal er wel afstoting optreden. Als altijd dezelfde stammen worden gebruikt weet je altijd zeker dat eenzelfde soort reactie zal optreden. Op deze manier kan de invloed van chronische afstoting worden onderzocht op het ontstaan van afwijkingen zoals bronchiolitis obliterans na longtransplantatie.

Ongeveer 15 jaar geleden is in Groningen door dr. K.W. Marck en dr. Jm. Prop een longtransplantatiemodel voor de rat onwikkeld, waarbij alleen de linker long wordt getransplanteerd. Vooral werk van Prop heeft ons inzicht in het mechanisme van de acute longafstoting enorm vergroot. Met dit longtransplantatie-model bleek het ook mogelijk langdurige overleving van allogeen getransplanteerde longen te bewerkstelligen na behandeling met cyclosporine, een immunosuppressivum. Deze getransplanteerde longen worden dus niet meer acuut afgestoten, maar blijken tot anderhalf jaar na transplantatie goed te kunnen functioneren. Dit is heel lang voor een laboratoriumrat, die meestal niet ouder dan 2 tot 3 jaar wordt. Met dit model hebben we de na longtransplantatie bij de mens de meest waarschijnlijke oorzaken voor het ontstaan van bronchiolitis obliterans onderzocht: chronische afstoting en virusinfecties.

2. Bronchiolitis obliterans na longtransplantatie in de rat

Chronische afstoting

In het eerste onderzoek (hoofdstuk 2.1) vonden we dat chronische afstoting bij ratten, die infectievrij werden gehouden, inderdaad schade aan de luchtwegen van allogeen getransplanteerde longen kan veroorzaken. Zoals gezegd worden allogeen getransplanteerde rattelongen die worden behandeld met cyclosporine niet meer acuut afgestoten. Dit wil echter niet zeggen dat de getransplanteerde long volledig wordt geaccepteerd. Het bleek namelijk dat er in deze allogeen getransplanteerde longen een vorm van bronchiolitis ontstond die in bepaalde opzichten leek op bronchiolitis obliterans bij de mens. In eerder onderzoek hebben we aangetoond dat vroeg na transplantatie veel lymfocyten vanuit de bloedbaan van ontvanger naar de getransplanteerde long migreerden. Deze lymfocyten waren vooral aanwezig rond de bloedvaten en de luchtwegen van de long. Daarnaast vond op sommige plaatsen in de long op de epitheelcellen expressie plaats van zogenaamde klasse II weefselantigenen, wat een teken is dat er een ontstekingsproces gaande is in de buurt van deze epitheelcellen. De infiltraten rond de bloedvaten en de kleine luchtwegen verdwenen in de daaropvolgende 2 maanden grotendeels spontaan. Zes maanden na transplantatie bleven er echter kleine infiltraatjes van cellen in de grote luchtwegen bleven bestaan, die wezen op een chronisch afstotinsgsproces: lymfocyten van het T-helper type, ophopingen van (ontvanger) dendritische cellen (dit zijn cellen die afwijkende cellen aan het afweersysteem presenteren zodat dit geactiveerd kan worden), en klasse II weefselantigenen op het epitheel. Het epitheel was bij deze infiltraten lokaal beschadigd en stukjes littekenweefsel groeiden in de opening van de luchtweg. Het proces bleef daarna stabiel of slechts langzaam progressief tot ten minste anderhalf jaar na transplantatie en bleef gedurende deze tijd beperkt tot de grote luchtwegen. Dat deze luchtwegafwijkingen door chronische afstoting en niet door de gevolgen van de transplantatie werden veroorzaakt bleek uit het feit dat syngeen getransplanteerde longen anderhalf jaar na transplantatie volkomen normale luchtwegen hadden. Deze experimenten toonden overduidelijk aan dat chronische afstoting, zonder infectie, afwijkingen in getransplanteerde rattelongen veroorzaak.

Omdat brochiolitis obliterans zoals we die zien bij mensen echter veel ernstiger is èn vooral gelokaliseerd is in de kleine luchtwegen, wijzen deze resultaten er op dat er mogelijk een tweede factor is die samen met chronische afstoting de ernstige vorm van bronchiolitis obliterans veroorzaakt bij longtransplantatie patiënten. Omdat virusinfecties naast chronische rejectie steeds worden genoemd

als mogelijke oorzaak van bronchiolitis obliterans, hebben we in vervolgexperimenten het effect van een virale infectie in allogeen getransplanteerde longen onderzocht (hoofdstuk 2.2).

Virus infecties

Het bleek dat een virale luchtweginfectie de eerder waargenomen lichte vorm van bronchiolitis sterk verergerde. Dit toonden we aan door longen met chronische afstoting 6 maanden na transplantatie te besmetten met het Sendai luchtwegvirus (Parainfluenza type I). In normale longen veroorzaakt het Sendai virus een lichte vorm van bronchiolitis, die na 3 weken helemaal genezen is. In de getransplanteerde longen met chronische afstoting ontwikkelde zich echter binnen een week een ernstige bronchiolitis, met sterk beschadigd epitheel en een heftige ontsteking. Zowel het epitheel van de grote als de kleine luchtwegen was ernstig beschadigd. De bronchiolitis nam in ernst toe tot 21 dagen na de infectie en bleef gedurende de gehele periode van het experiment, 8 weken, aanwezig. Als gevolg van deze heftige ontstekingsreactie onstond een bronchiolitis obliterans die sprekend leek op die bij patiënten na longtransplantatie, met een duidelijke vernauwing van met name de kleine luchtwegen.

Ook bij deze experimenten bleek dat syngeen getransplanteerde longen op dezelfde manier reageren als normale, niet-getransplanteerde longen, want het beloop van de infectie was exact hetzelfde als in de normale longen. Dit onderzoek is het eerste experimentele bewijs, dat ernstige bronchiolitis obliterans in longtransplantaten wordt veroorzaakt door een combinatie van chronische afstoting en virale infecties. Maar wat gaat er nu mis tijdens het gelijktijdig optreden van chronische afstoting en virale infectie? Om hierover meer te weten te komen hebben we in het volgende onderzoek meer gedetailleerd gekeken naar de ontstekingsreactie in getransplanteerde longen met infectie.

Ontstekingsreacties

De ernstige bronchiolitis obliterans na virusinfectie in de allogeen getransplanteerde longen bleek te worden veroorzaakt door een abnormaal sterke ontstekingsreactie vroeg na infectie (hoofdstuk 2.3). Tijdens deze heftige ontstekingsreactie waren zeer veel geactiveerde macrofagen en neutrofiele granulocyten aanwezig, die in de syngeen getransplanteerde long na infectie in veel mindere mate aanwezig waren.Vooral tijdens deze heftige ontstekingsreactie in de allogeen getransplanteerde long wordt het epitheel aantast, met als gevolg daarvan een sterke verlittekening van de luchtwegen. Verder bleek dat de cellen die normaal een belangrijke rol spelen in de bescherming tegen virusinfecties, de

cytotoxische T lymfocyten, veel trager reageerden in deze longen dan in syngeen getransplanteerde en niet-getransplanteerde longen: het duurde ongeveer 3 weken voordat zij in normale hoeveelheden in de allogeen getransplanteerde longen aanwezig waren. Maar op dat moment was het kwaad door de infectie al geschied. Verder bleek dat er bijna geen B lymfocyten, de cellen die antilichamen produceren, naar de luchtwegen migreerden, wat in de syngeen getransplanteerde longen wel het geval was. Het lijkt er dus op dat de verdediging tegen virussen gestoord is in allogeen getransplanteerde longen. Dit zou ook kunnen verklaren waarom er zoveel infecties optreden in getransplanteerde longen bij mensen. Omdat er weinig bekend is over het functioneren van het afweersysteem van de getransplanteerde long, hebben we in het tweede gedeelte van ons onderzoek gekeken hoe het met dit afweersysteem is gesteld na longtransplantatie.

3. Gestoorde afweer tegen infecties na longtransplantatie in de rat

Tijdens elke ademhaling bereiken allerlei deeltjes de long, variërend van stofdeeltjes tot virussen en bacteriën. Om niet ten onder te gaan aan deze invasie van pathogenen zijn onze longen uitgerust met een zeer uitgebreid afweersysteem (hoofdstuk 3.1). Bij gezonde mensen zal dit afweersysteem de ingeademende pathogenen onschadelijk maken. Deze afweermechanismen variëren van de aspecifieke hoestreflex tot complexe immunologische reacties waarbij antilichamen worden geproduceerd die specifiek zijn gericht tegen het ingeademde antigeen. Deze antilichaamproductie vindt plaats in het lymfoïede weefsel, dat op verschillende plaatsen binnen en buiten de long aanwezig is. Met name de antilichamen die lokaal geproduceerd worden in het lymfoïede weefsel van de long, in het BALT (bronchus-associated lymphoid tissue), zijn erg belangrijk als eerste verdedigingslinie tegen virussen en bacteriën. Daarnaast is antilichaamproductie in de lymfeklieren vlakbij de long belangrijk voor bescherming van de long en de rest van het lichaam.

In dit hoofdstuk beschrijven we het onderzoek naar de antilichaamreactie in het bloed en lokaal in de long, tegen twee antigenen die in de getransplanteerde long waren gebracht : schape-erythrocyten (rode bloedcellen) en het Sendai virus.

Antilichaamreactie tegen schape-erythrocyten

De antilichaamreactie in het bloed tegen schape-erythrocyten die in de long waren gespoten, bleek gestoord na longtransplantatie. Opvallend was echter dat dit alleen kort na transplantatie het geval was (hoofdstuk 3.2). Een maand na transplantatie kon een normale antilichaamreactie worden opgewekt in de

allogeen getransplanteerde ratten. Deze tijdelijke verstoring van de antilichaamreactie bleek het gevolg te zijn van het doorsnijden tijdens de transplantatieprocedure, van de lymfevaten die naar de lymfeklieren van de long lopen.

In experimenten waarbij we alleen de lymfevaten doorsneden zonder de long te transplanteren bleek dat na ongeveer een week deze zich spontaan herstelden en parallel hieraan bleek de antilichaamproductie weer op gang te komen (hoofdstuk 3.3). Na 2 tot 4 weken was de antilichaamreactie weer helemaal genormaliseerd. Gelukkig herstellen deze lymfebanen zich dus vrij snel, zodat de antilichaamreactie weer normaal verloopt.

Deze eerste twee weken na transplantatie zijn de longen dus gevoelig voor infecties doordat de lymfevaten zijn doorgesneden. Het is daarom belangrijk om in deze vroege periode na transplantatie extra alert te zijn op het optreden van infecties. Het lijkt ons dan ook belangrijk om in deze kwetsbare periode sterke preventieve maatregelen te nemen tijdens allerlei diagnostische ingrepen, zoals het nemen van longbiopten, waarvan bekend is dat ze een belangrijke bron van infectie kunnen zijn.

Antilichaamreactie tegen Sendaivirus

Ook de antilichaamreactie, zowel in het bloed als lokaal in het BALT, tegen het Sendaivirus bleek verminderd na allogene longtransplantatie (hoofdstuk 3.4). Het grote verschil met de schape-erythrocyten was echter dat deze stoornis blijvend was. Het meest opvallend was het lage aantal antilichaamproducerende cellen in het BALT na infectie in deze allogene longtransplantaten. Door deze sterk verminderde antilichaamproductie was het virus veel langer aanwezig in deze longen. Dit was in scherpe tegenstelling met de syngeen getransplanteerde en niet-getransplanteerde longen, waar het aantal antilichaamproducerende cellen enorm toenam na infectie en het virus slechts enkele dagen in de long aantoonbaar was. Het lijkt er dus op dat het BALT door de allotransplantatie beschadigd raakt, waardoor het vervolgens niet normaal kan functioneren na infectie.

Daarom hebben we in vervolgexperimenten in longtransplantaten gekeken naar 3 belangrijke voorwaarden voor een normale functie van het BALT: de structuur, de opname van antigenen vanuit de luchtwegen en de migratie van lymfocyten vanuit de bloedbaan naar het BALT (hoofdstuk 3.5). Het bleek dat het BALT in de allogeen getransplanteerde longen op alle drie de voorwaarden ernstig was aangetast. Het BALT was veel kleiner, bevatte veel minder lymfocyten dan normaal en was voor een groot gedeelte vervangen door littekenweefsel.

Verder bleek dat opname van deeltjes vanuit de luchtwegen gestoord was en dat de migratie van lymfocyten naar het BALT sterk was verminderd. Deze sterke beschadiging verklaart het uitblijven van antilichaamproductie na infectie in deze longen. Het is het meest waarschijnlijk dat het BALT beschadigd is door afstoting, omdat het BALT in de syngeen getransplanteerde longen helemaal normaal was. Ook in eerder onderzoek van Prop is al aangetoond dat het BALT een eerste doelwit van onbehandelde acute afstoting is en het lijkt er op dat dit helaas ook het geval is als acute afstoting wordt behandeld met cyclosporine. In hoofdstuk 5 wordt verder ingegaan op mogelijke preventieve maatregelen die schade aan het BALT in allogeen getransplanteerde longen kunnen voorkomen.

4. De immuunrespons na longtransplantatie bij de mens

Uit bovenstaande blijkt dat bronchiolitis obliterans na longtransplantatie in de rat ontstaat door het ontsporen van ontstekings-, en afweerreacties na afstoting en infectie in de getransplanteerde long. Hoewel resultaten van proefdieronderzoek niet zonder meer vertaald mogen worden naar de klinische situatie, gaan we ervan uit dat bronchiolitis obliterans na longtransplantatie bij de mens door dezelfde ontspoorde reacties ontstaat als bij de rat (hoofdstuk 4.1). Vroege opsporing van deze reacties zou een snelle behandeling en daardoor wellicht preventie van bronchiolitis obliterans mogelijk maken. Ontstekings-, en afweerreacties na afstoting en infectie spelen zich af in het longweefsel en kunnen daarom het best worden opgespoord in longweefsel dat verkregen is door een biopt te nemen uit de getransplanteerde long. Dit kan op een betrouwbare en veilige manier gebeuren met zogenaamde transbronchiale biopten. Hierbij worden kleine hapjes uit de getransplanteerde long genomen, waarin als het goed is stukjes van het luchtwegepitheel, bloedvaatjes en ander longweefsel aanwezig is. Het blijkt echter in de praktijk dat lang niet altijd voldoende weefsel in deze biopten aanwezig is, waarbij vooral het luchtwegepitheel vaak ontbreekt. Hierdoor is het moeilijk of onmogelijk om een goede indruk te krijgen van de schade aan het longweefsel. Wel zou het specifiek aantonen van verschillende soorten cellen die betrokken zijn bij ontstekings- en afweerreacties, zoals de eerdere genoemde macrofagen en lymfocyten, een aanwijzing kunnen zijn voor de mogelijke beschadiging van de luchtwegen en het longweefsel.

In een klinische studie hebben we gekeken naar de aanwezigheid van deze cellen tijdens vroege fasen van acute en chronische longafstoting na hart-long transplantatie. We vonden dat detectie van specifieke cellen inderdaad zinvol kan zijn om een beter inzicht te krijgen in de ontstekings- en afweerreacties in longtransplantaten, zelfs als er weinig weefsel beschikbaar is in de biopten (hoofstukt 4.2). In de vroege fasen van acute en chronische afstoting waren

infiltrerende cellen aanwezig rond de bloedvaten, die hetzelfde waren als de infiltrerende cellen in de luchtwegen. De ontstekingsreactie tijdens acute afstoting was heftiger dan tijdens chronische afstoting, met een opvallende aanwezigheid van geactiveerde macrofagen.

Deze studie toont aan dat opsporing van specifieke cellen in longbiopten meer inzicht kan geven in de immunologische processen die gaande zijn in getransplanteerde longen, zelfs als er weinig weefsel beschikbaar is. En wat belangrijk is in het kader van de bevindingen in de voorgaande hoofdstukken: het lijkt er inderdaad op dat zich vergelijkbare reacties afspelen na longtransplantatie bij de mens als de reacties die we vonden na longtransplantatie bij de rat. Op deze manier kunnen proefdieronderzoek en klinisch onderzoek samen meer inzicht geven in de mechanismen die leiden tot het ontstaan van bronchiolitis obliterans na longtransplantatie.

5. Oorzaken van bronchiolitis obliterans in longtransplantaten.

Concluderend komen we tot de volgende hypothese over het ontstaan van bronchiolitis obliterans na longtransplantatie: chronische afstoting veroorzaakt na transplantatie geringe schade aan de luchtwegen van de getransplanteerde long. Belangrijker is echter dat dezelfde afstoting het lokale afweersysteem in de getransplanteerde long beschadigt, zodanig dat dit niet meer normaal kan functioneren tegen infecties. Als de long dan geïnfecteerd wordt door een luchtwegvirus, veroorzaakt dit een abnormaal heftige en langdurige bronchiolitis met sterke verlittekening van de kleine luchtwegen: het beeld van bronchiolitis obliterans. Andere factoren die ook ontstekingsreacties in de getransplanteerde long veroorzaken, zoals ischemie, kunnen dit hele proces nog eens versterken.

Preventie van bronchiolitis obliterans zou zich moeten begeven op twee terreinen. Ten eerste agressieve onderdrukking van afstoitng in een vroeg stadium na transplantatie om het lokale afweersysteem in de long intact te laten. Ten tweede het onderdrukken van abnormale heftige ontstekingsreacties in de getransplanteerde long na infecties. Kennelijk schieten de huidige behandelingsmethoden in deze opzichten tekort. Afstoting wordt permanent met meerdere immunosuppressiva behandeld. Hierbij worden ook corticosteroïden gebruikt, die sterke ontstekingsremmers zijn. Het is echter voorstelbaar dat deze medicijnen uit voorzichtigheid vaak te laat of in een te lage dosering worden gegeven, in verband met de kans op ernstige bijwerkingen. Bovendien kan de noodzakelijke locale concentratie onvoldoende hoog zijn om de heftige ontstekingsreactie te onderdrukken. Een manier om deze problemen te voorkomen is wellicht het lokaal toedienen van medicijnen in de

getransplanteerde long, zoals ook bij de CARA-behandeling steeds meer medicijnen via inhalatie worden ingenomen. Dit lijkt ook na longtransplantatie een goede optie te zijn, aangezien de de reacties die leiden tot bronchiolitis obliterans, sterk gelokaliseerd zijn in en rond de luchtwegen, en zodoende gemakkelijk bereikbaar voor geïnhaleerde stoffen. Zowel de afstoting als de ontsteking zouden op deze manier behandeld kunnen worden.

Een andere, nog experimentele, behandeling van het longtransplantaat om afstoting, met name van het BALT te voorkomen, is de bestraling van de donorlong vòòr uitname van de long uit de donor, waardoor de sterk immunogene lymfocyten in het BALT van de te transplanteren long verdwijnen. De structuur van het BALT zou in deze bestraalde longen beter intact kunnen blijven en zelfs repopulatie met lymfocyten van de ontvanger mogelijk maken. Mogelijk dat hierdoor de functie van het BALT hersteld kan worden en een normale afweer tegen infecties ontstaat.

Slot

Uit het bovenstaande verhaal blijkt dat transplantatie van een long meer inhoudt dan alleen de operatie. De patiënt is na de transplantatie nog lang niet van zijn problemen, laat staan van zijn dokters af. Na de transplantatie gaat het een tijd goed met de getransplanteerde long maar dan ontstaan er nieuwe problemen in de gedaante van bronchiolitis obliterans. Hoewel aanvankelijk onontwarbaar, beginnen we langzaam de kluwen van oorzaken van deze verraderlijke aandoening te ontrafelen. Hierdoor kunnen rationele therapieën ontstaan ter voorkoming en zonodig ter behandeling van bronchiolitis obliterans in getransplanteerde longen.

6.2 Résumé en français

1. Développement de la transplantation du poumon

La transplantation du poumon a connu un tel essor ces dix dernières années qu'elle peut être réalisée aujourd'hui avec beaucoup de succès sur des personnes souffrantes de graves maladies pulmonaires. La plupart des receveurs de transplants pulmonaires peuvent reprendre une vie normale et nombreux sont ceux qui ont même repris leurs activités professionnelles. Voilà ce qui semble très optimiste, mais les apparences sont, ici encore, trompeuses. Cette dernière décennie les résultats au cours de la première année après une transplantation pulmonaire ont montré une très nette amélioration; cependant, après cette première année la fonction du poumon transplanté subit une détérioration telle qu'un nombre important d'opérés n'y survivent pas. La période de survie de cinq ans- qui, en médecine, permet de mesurer le succès d'un traitement- est obtenue en moins de 50% des cas, tous types de transplantation confondus. C'est dire que cinq ans après la transplantation pulmonaires seulement la moitié des personnes transplantées sont encore en vie. Il est apparu que presque tous les opérés qui sont morts ont manifesté une réduction des fonctions pulmonaires à la suite d'un rétrécissement des bronchioles dû à une inflammation. Cette affection est appelée bronchiolite obliterans. L'origine de la bronchiolite obliterans après transplantation pulmonaire n'est malheureusement pas très claire et par conséquent difficile à traiter.

La bronchiolite obliterans débute par des lésions de l'épithélium, le revêtement des voies respiratoires, aussi bien chez les transplantés que chez les non-transplantés qui développent une bronchiolite obliterans. Chez les non-transplantés la bronchiolite obliterans peut survenir après des lésions de l'épithélium à la suite d'infections virales ou de l'inhalation de gaz toxiques. Cette inflammation, née dans les voies respiratoires après une lésion de l'épithélium, est identique dans la plupart des cas, même si les causes sont différentes. Après une première lésion de l'épithélium le corps réagit en développant une inflammation pour limiter les dégâts et pour les réparer. Lors de cette réaction des cellules de différentes sortes se mettent en migration via le sang vers l'endroit lésé. Macrophages, granulocytes neutrophiles et, dans certains cas, lymphocytes se dirigent en masse vers la lésion pour y détruire les cellules atteintes de l'épithélium de sorte que de nouvelles cellules de l'épithélium puissent naître, jusqu'à la disparition complète de la lésion. Ce processus de destruction et de réparation est appelé une réaction inflammatoire, qui prend fin quand tout a été réparé. Dans le cas de la bronchiolite obliterans l'inflammation cependant reste trop active et il n'y a pas de vraie phase de réparation. Cette inflammation persistante est à l'origine de la formation de tissus cicatriciels surtout, les cellules de l'épithélium étant presque absentes. Ce tissu cicatriciel est très rigide de sorte

que les voies respiratoires continuent à se rétrécir.

La bronchiolite obliterans est une maladie assez rare chez les non-transplantés. Sa forte fréquence après les transplantations pulmonaires reste inexpliquée. Actuellement il est supposé que l'inflammation dans les voies respiratoires est provoquée par une sorte de réactivité immunitaire chronique du receveur face au poumon transplanté, il s'agit donc d'un rejet chronique, et par des infections (virales) du poumon, très fréquentes après transplantation.

Aujourd'hui le rejet chronique est considéré comme la principale cause de la bronchiolite obliterans. Aussi la ISHLT (International Society for Heart and Lung Transplantation) a classé la bronchiolite obliterans dans le groupe des "chronic air way rejection" (rejets chroniques dans les voies respiratoires). Deux expériences cliniques ont démontré clairement qu'il y a un lien entre bronchiolite obliterans et rejet. La première a montré que des cellules du receveur peuvent être localisées dans le poumon transplanté qui sont réactives aux antigènes tissulaires du transplant et ce avant que la bronchiolite obliterans ne devienne manifeste. La deuxième expérience décrit une présence accrue de la bronchiolite obliterans dans les poumons ayant connu plusieurs périodes de rejet aigu.

Si le lien entre l'infection et la bronchiolite obliterans est sans cesse signalé, son mécanisme n'en est pas moins opaque. C'est notamment l'infection du CMV (Cytomégalovirus) qui est donnée comme la principale cause de la bronchiolite obliterans; il est cependant de plus en plus clair que d'autres infections (virales) du poumon et des voies respiratoires peuvent provoquer une bronchiolite obliterans. Une infection apparemment anodine des voies respiratoires peut amener une réduction de la fonction du transplant pulmonaire qui ne sera pas rétablie par la suite. Dans les biopsies pulmonaires on voit d'abord une bronchiolite a-spécifique qui se développe ensuite progressivement pour devenir une bronchiolite obliterans évidente. Bien que le rejet et l'infection soient de possibles causes de la bronchiolite obliterans dans les transplants pulmonaires, leur apparition et leur évolution chez des cas individuels restent imprévisibles. Souvent il est donc incertain quelle est la cause principale de la bronchiolite obliterans dans le poumon transplanté.

Cette compréhension imparfaite de l'origine de la bronchiolite obliterans risque de créer une impasse qui fait obstacle au développement des transplantations pulmonaires chez l'homme. Des recherches effectuées sur des animaux seraient susceptibles de fournir une meilleure compréhension de l'origine de la bronchiolite obliterans après transplantation pulmonaire. Celles-ci ont le grand avantage de permettre une recherche isolée et combinée des différents facteurs censés provoquer une bronchiolite obliterans dans le poumon

transplanté. Ainsi, grâce au croisement consanguin, les recherches sur les rats ont donné des lignées dont les individus ont les mêmes caractéristiques tissulaires. Si dans une transplantation le donneur et le receveur appartiennent à la même lignée il n'y aura pas de rejet de l'organe transplanté, parce que leurs antigènes tissulaires sont identiques: il s'agit d'une transplantation syngène. Par contre, si le donneur et le receveur appartiennent à des lignées différentes-la transplantation allogène-le rejet aura lieu. L'emploi permanent des mêmes lignées garantit toujours le même type de réaction. Ainsi peutêtre étudiée l'influence du rejet chronique sur l'apparition d'anomalies comme la bronchiolite obliterans après transplantation pulmonaire.

Il y a 15 ans les docteurs K.W.Marck et Jm.Prop ont développé à Groningue (Pays-Bas) un modèle de transplantation pulmonaire pour le rat où seul le poumon gauche est transplanté. Ce sont notamment les travaux de Prop qui ont permis de faire un grand pas en avant dans la compréhension du mécanisme qui est à l'origine du rejet aigu du poumon transplanté. Ce modèle de transplantation pulmonaire a également permis de réaliser une survie prolongée de transplants pulmonaires allogènes après un traitement au cyclosporine, un immunodépresseur.

Ces poumons transplantés ne subissent donc plus de rejet aigu, mais présentent un fonctionnement adéquat jusqu'à dix-huit mois après transplantation, ce qui est très long pour un rat de laboratoire dont la durée de vie est de 2 à 3 ans dans la plupart des cas. Nous avons appliqué ce modèle à l'homme transplanté pour étudier les causes les plus probables de l'apparition de la bronchiolite obliterans: rejet chronique et infections virales.

2. Bronchiolite obliterans après transplantation pulmonaire dans le rat

Rejet chronique

La première recherche (ch.2.1) a démontré que le rejet chronique chez les rats, sans infections, peut en effet provoquer des lésions aux voies respiratoires des transplants pulmonaires allogènes. Nous avons déjà constaté que les transplants pulmonaires allogènes des rats traités au cyclosporine ne présentent plus de rejet aigu. Ce qui n'implique pas cependant une acceptation complète du poumon transplanté. Il a été établi que dans ces transplants pulmonaires allogènes une forme de bronchiolite s'est manifestée qui, sous certains aspects, ressemblait à celui des humains. Dans une recherche antérieure nous avions démontré la migration d'un grand nombre de lymphocytes depuis les vaisseaux sanguins du receveur vers le poumon transplanté dans la première phase après

transplantation. Ces lymphocytes étaient surtout présents autour des vaisseaux sanguins et des voies respiratoires du poumon. De plus, à certains endroits dans le poumon, sur les cellules épithéliales, se manifestaient des antigènes tissulaires dites de la classe II, signe qu'un processus inflammatoire est en cours dans le voisinage de ces cellules de l'épithélium. Ces infiltrats autour des vaisseaux sanguins et des petites voies respiratoires disparaissaient pour la plupart spontanément dans les deux mois suivants. Six mois après transplantation cependant de petits infiltrats de cellules se maintenaient dans les grandes voies respiratoires, ce qui indiquait un processus de rejet chronique: lymphocytes du type T-activeur, concentrations de cellules dendrites (receveur)-ce sont des cellules qui présentent des cellules étrangères au système immunitaire de façon à l'activer-et des antigènes tissulaires de la classe II sur l'épithélium. Lors de ces infiltrations l'épithélium avait subi localement des lésions et des particules de tissu cicatriciel poussaient dans l'ouverture de la voie respiratoire. Par la suite ce processus restait stable ou ne montrait qu'une lente progression jusqu'au moins 18 mois après transplantation et se limitait au cours de cette période aux grandes voies respiratoires. Nous avons pu constater que ces anomalies des voies respiratoires sont dues au rejet chronique et non pas aux séquelles de la transplantation, les transplants pulmonaires syngènes présentant des voies respiratoires parfaitement normales 18 mois après transplantation. Ces expériences ont démontré de façon concluante que le rejet chronique, sans inflammation, cause des anomalies dans les poumons de rats transplantés.

Or, la bronchiolite obliterans que nous relevons chez l'homme étant beaucoup plus grave et de surcroît localisé surtout dans les petites voies respiratoires, ces résultats signifient qu'il y a probablement un deuxième facteur qui, en collusion avec le rejet chronique, est à l'origine de cette version grave de la bronchiolite obliterans chez les sujets transplantés. Comme, outre le rejet chronique, les infections virales sont toujours signalées en tant qu'une des causes probables de la bronchiolite obliterans, nous avons étudié dans des expériences complémentaires les effets d'une infection virale dans les transplants pulmonaires allogènes. (ch.2.2)

Infection virale

Nous avons constaté qu'une infection virale des voies respiratoires entraînait une aggravation de la bronchiolite observée plus tôt dans un stade moins préoccupant. A cet effet nous avons contaminé des poumons présentant un rejet chronique six mois après transplantation avec le virus des voies respiratoires Sendai(Parainfluenza Type I). Dans les poumons sains le virus Sendai provoque une forme légère de bronchiolite qui est entièrement guérie au bout de trois

semaines. Cependant, dans les poumons transplantés à rejet chronique se développait en moins de huit jours une bronchiolite grave avec une forte lésion épithéliale et une inflammation virulente. L'épithélium des grandes ainsi que des petites voies respiratoires était gravement atteint. La bronchiolite présentait une évolution aggravante 21 jours après l'infection et se maintenait pendant toute la période de l'expérience, qui durait huit semaines. Cette réaction inflammatoire très violente donnait naissance à une bronchiolite obliterans qui présentait une très forte analogie avec celle des sujets ayant subi une transplantation pulmonaire, avec un rétrécissement apparent des petites voies respiratoires notamment.

Ces expériences ont également révélé que les transplants pulmonaires syngènes réagissent de la même façon que les poumons normaux, non transplantés, car l'évolution de l'infection était exactement la même que celle des poumons normaux. Cette recherche fournit donc la première preuve expérimentale qu'une bronchiolite grave dans les transplants pulmonaires est causé par une combinaison de rejet chronique et d'infections virales. La question est de savoir ce qui tourne mal quand il y a apparition simultanée de rejet chronique et d'infections virale. Pour tenter de répondre à cette question nous avons analysé en détail la réaction inflammatoire dans les poumons transplantés présentant une infection.

Réactions inflammatoires

La bronchiolite obliterans grave après infection virale dans le transplant pulmonaire allogène était causé par une réaction inflammatoire anormalement forte tôt après infection (ch.2.3). Pendant cette inflammation virulente nous avons relevé la présence d'un très grand nombre de macrophages activés et de granulocytes neutrophiles, qui étaient beaucoup moins nombreux dans le transplant pulmonaire syngène après infection. C'est surtout pendant cette réaction inflammatoire virulente dans le transplant pulmonaire allogène que l'épithélium subit des lésions, qui entraînent une forte cicatrisation des voies respiratoires. Ensuite il est apparu que les cellules qui normalement jouent un rôle important dans la protection contre les infections virales, les lymphocytes T cytotoxiques, avaient dans ces poumons une réaction beaucoup plus lente que dans les transplants syngènes et les poumons non transplantés: il fallait attendre environ trois semaines avant qu'ils ne se manifestent en quantités normales dans les transplants pulmonaires allogènes. Mais alors le mal dû à l'infection était déjà fait. D'ailleurs il n'y avait guère de lymphocytes, les cellules qui produisent les anticorps, en migration vers les voies respiratoires, comme c'était le cas dans les transplants pulmonaires syngènes. On dirait donc que la défense anti-virale est

perturbée dans les transplants pulmonaires allogènes. Cela expliquerait la forte fréquence d'infections dans les poumons transplantés chez l'homme. Comme nous savons encore peu du fonctionnement du mécanisme de défense du poumon transplanté, cela fera l'objet de la deuxième partie de notre recherche.

3. Défense perturbée contre les infections dans les poumons transplantés

Chaque inspiration introduit dans le poumon toutes sortes de particules, des grains de poussière, mais aussi des virus et des bactéries. Afin de résister à cette invasion des éléments pathogènes, les poumons sont équipés d'un système de défense très sophistiqué (ch.3.1). Chez les sujets sains ce système de défense détruira les particules pathogènes inspirés. Ces mécanismes de défense vont de l'a-spécifique acteréflexe de la toux aux réactions immunologiques complexes qui produisent des anticorps spécifiques élaborés en réaction à l'introduction de l'antigène. Cette production d'anticorps se fait dans le tissu lymphoïde présent dans plusieurs régions à l'intérieur et à l'extérieur du poumon. Ce sont notamment les anticorps produits localement dans le tissu lymphoïde du poumon, dans le BALT (bronchus-associated lymphoid tissue), qui se trouvent en première ligne de défense contre les virus et les bactéries. Par ailleurs, la production d'anticorps dans les ganglions lymphatiques près du poumon est importante pour la protection du poumon et le reste du corps.

Dans ce chapitre nous donnons une description de la recherche sur la réponse immunitaire humorale dans le sang et localement dans le poumon, à l'introduction de deux antigènes dans le poumon transplanté: les érythrocytes de mouton (globules rouges) et le virus Sendai.

Réponse immunitaire humorale aux érythrocytes de mouton

La réponse immunitaire humorale dans le sang aux érythrocytes de mouton injectés dans le poumon s'est avérée perturbée après transplantation pulmonaire. Il était cependant curieux de constater que cette perturbation n'était que de courte durée suite à la transplantation (ch.3.2). Un mois après transplantation une réponse immunitaire humorale normale pouvait être provoquée dans les rats ayant subi une transplantation allogène. Cette perturbation temporaire était due à la section, au cours de la procédure de transplantation, des vaisseaux lymphatiques qui vont aux ganglions lymphatiques du poumon.

Les expériences au cours desquelles seulement les vaisseaux lymphatiques avaient été sectionnés sans transplantation pulmonaire, ont démontré que ceux-ci se rétablissaient spontanément au bout d'une semaine et qu'en même temps la production d'anticorps avait repris (ch.3.3). Après 2 à 4 semaines la réponse

immunitaire humorale s'était entièrement normalisée. C'est donc avec satisfaction que nous constatons que ces vaisseaux lymphatiques se reconstituent assez vite au point d'assurer une reprise normale de la réponse immunitaire humorale. Les deux premières semaines après transplantation les poumons sont donc réceptifs aux infections puisque lesvaisseaux lymphatiques ont été sectionnés. Voilà pourquoi il est important dans cette première période après transplantation d'être particulièrement vigilant à l'apparition d'infections. Pendant cette phase précaire de mesures préventives vigoureuses s'imposent au cours de toutes sortes d'interventions diagnostiques, telles que la prise de biopsies pulmonaires, dont on sait qu'elles induisent facilement des infections.

Réponse immunitaire humorale au virus Sendai

Nous avons vu que la réponse immunitaire humorale au virus Sendai, aussi bien dans le sang que localement dans le BALT, s'est également révélée amoindrie après implantation d'un poumon allogène (ch.3.4). Contrairement aux érythrocytes de mouton, cette perturbation était persistante. Le plus frappant était la quantité réduite de cellules élaborant des anticorps dans le BALT après infection dans ces transplants pulmonaires allogènes. Cette forte réduction de la production d'anticorps avait permis une présence prolongée du virus dans ces poumons. C'était là une très nette différence avec les transplants syngènes et les poumons non transplantés dans lesquels le nombre de cellules productrices d'anticorps avait énormément augmenté après infection et où le virus n'était détectable que pendant quelques jours. Tout porte donc à supposer que le BALT est abîmé par cette allotransplantation, œ qui entraîne un dysfonctionnement après infection.

Dans des expériences complémentaires nous avons donc été amené à étudier dans les transplants pulmonaires les trois principales conditions permettant une fonction normale du BALT: la structure, la pénétration d'antigènes depuis les voies aériennes et la migration de lymphocytes depuis les vaisseaux sanguins vers le BALT. Dans les trois cas nous avons constaté une forte agression contre le BALT dans les transplants allogènes. Le BALT était beaucoup plus petit, contenait moins de lymphocytes que normalement et était remplacé en grande partie par un tissu cicatriciel. Ensuite la pénétration de particules depuis les voies aériennes était perturbée et la migration de lymphocytes vers le BALT était fort réduite. Cette lésion considérable fournit une explication à la suspension de production d'anticorps après infection dans ces poumons. C'est l'hypothèse d'une lésion du BALT à cause du rejet qui nous semble la plus probable, le BALT étant parfaitement normal dans les transplants syngènes. Une étude de Prop avait

également démontré déjà que le BALT est la première cible du rejet aigu non traité et tout porte donc à croire que c'est malheureusement aussi le cas si le rejet aigu est traité au cyclosporine. Dans le chapitre 5 nous préciserons les mesures préventives susceptibles de préserver le BALT de lésions dans les transplants pulmonaires allogènes.

4. La réponse immunitaire après transplantation du poumon chez l'homme

Nous avons démontré dans les chapitres précédents que la bronchiolite obliterans après transplantation du poumon dans le rat est due à un dérèglement des réactions inflammatoires et immunitaires après rejet et infection dans le poumon transplanté. Tout en exprimant nos réserves quant à une transposition pure et simple des résultats obtenus sur des animaux de laboratoire vers la situation clinique, nous admettons que la bronchiolite obliterans après transplantation du poumon humain est causée par les mêmes réactions déréglées que celles constatées chez le rat (ch.4.1). Un dépistage de ces réactions dans un stade initial devrait permettre un traitement d'urgence et donc probablement la prévention de la bronchiolite obliterans. Ces réactions inflammatoires et immunitaires après rejet et infection ont lieu dans le tissu pulmonaire. La meilleure façon de les dépister est encore de faire une biopsie sur le poumon transplanté. Cette intervention est sûre et efficace grâce aux biopsies dites transbronchiales et consiste à prélever de petits fragments du poumon transplanté où devraient se trouver des particules de l'épithélium aérien, des vaisseaux sanguins et d'autres tissus pulmonaires. Cependant, nous constatons que souvent la quantité de tissu fournie par ses biopsies est insuffisante, l'épithélium aérien notamment faisant fréquemment défaut. Il est donc difficile, voireimpossible de se faire une idée précise des lésions subies par le tissu pulmonaire. Par contre, l'identification de différentes sortes de cellules impliquées dans les réactions inflammatoires et immunitaires, telles les macrophages et les lymphocytes précités, pourrait indiquer une éventuelle lésion des voies respiratoires et du tissu pulmonaire.

Une étude clinique a permis de regarder de plus près la présence de ces cellules pendant les premières phases du rejet aigu et chronique après transplantation cardio-pulmonaire. Nous avons trouvé que l'identification de ces cellules spécifiques est utile car elle permet une meilleure compréhension des réactions inflammatoires et immunitaires dans les transplants pulmonaires, même s'il n'y a que peu de tissu disponible dans les biopsies (ch.4.2). Dans les premiers stades du rejet aigu et chronique nous avons localisé autour des vaisseaux sanguins des infiltrations de cellules identiques à celles qui s'étaient infiltrées dans les voies respiratoires. La réaction inflammatoire pendant le rejet aigu était

plus violente que lors du rejet chronique et était accompagnée d'une présence remarquable de macrophages activés.

Cette étude fait apparaître que le dépistage de cellules spécifiques dans les biopsies pulmonaires permet une meilleure compréhension des processus immunologiques qui se développent dans les poumons transplantés, même s'il y a peu de tissu disponible. Ensuite, par rapport aux conclusions des chapitres précédents, il est important de constater en effet que des réactions similaires à celles observées chez le rat semblent avoir lieu après transplantation pulmonaire chez l'homme. Ainsi les résultats conjugués de la recherche sur les animaux de laboratoire et de la recherche clinique permettent de mieux saisir les mécanismes provoquant la bronchiolite obliterans après transplantation pulmonaire.

5. Les causes de la bronchiolite obiterans dans les transplants pulmonaires

Nous pouvons conclure en avançant l'hypothèse suivante sur l'origine de la bronchiolite obliterans après transplantation pulmonaire: le rejet chronique provoque après transplantation une légère lésion des voies aériennes du poumon transplanté. Cependant il est plus important de constater que le même rejet entraîne une détérioration de la défense immunitaire locale du poumon transplanté, qui entrave son fonctionnement normal contre les infections. Si le poumon est infecté par un virus des voies aériennes, cette infection provoque un bronchiolite anormalement virulent et prolongé avec une forte cicatrisation des petites voies respiratoires: la manifestation de la bronchiolite obliterans. D'autres facteurs qui causent également des inflammations dans le poumon transplanté, tels que l'ischémie, sont susceptibles d'accentuer ce processus.

La prévention de la bronchiolite obliterans devrait s'appliquer à deux domaines. Le premier est celui de la répression agressive du rejet dans un premier stade après transplantation pour préserver le système immunitaire local du poumon. Le second est celui de la répression de réactions inflammatoires anormalement virulentes dans le poumon transplanté après infections. A cet effet, les méthodes de traitement actuellement en vigueur s'avèrent insuffisantes. Le rejet est traité en permanence avec plusieurs immunodépresseurs à la fois, dont les corticostéroïdes, qui ont une action anti-inflammatoire très forte. On peut s'imaginer cependant que, par prudence, ces médicaments sont souvent administrés trop tard ou en dosage insuffisant vu le risque d'effets secondaires. De plus, la concentration locale peut être trop peu élevée pour réprimer l'inflammation virulente. Afin d'éviter ces problèmes on pourrait appliquer localement des médicaments dans le poumon transplanté à l'exemple du traitement de la B.P.C.O. (broncho-pneumopathie chronique obstructive) où l'on

a de plus en plus recours à l'inhalation de médicaments. Après transplantation pulmonaire cette méthode paraît indiquée, les réactions qui provoquent la bronchiolite obliterans étant fortement concentrées dans les voies respiratoires et leurs alentours, qui sont facilement accessibles aux matières inhalées. Le rejet et l'inflammation pourraient être traités ainsi.

Un autre traitement, bien qu'expérimental encore, du transplant pulmonaire pour prévenir le rejet, notamment du BALT, est l'irradiation du poumon à réimplanter avant l'enlèvement du donneur pour éliminer les lymphocytes fort immunogènes dans le BALT du poumon à transplanter. La structure du BALT pourrait mieux se maintenir dans ces poumons irradiés et rendre possible une repopulation avec des lymphocytes du receveur, ce qui devrait rétablir la fonction du BALT permettant une réponse immunitaire normale.

Fin

Dans les pages précédentes nous pensons avoir suffisamment démontré que la transplantation d'un poumon ne s'arrête pas à l'opération proprement dite. Après la transplantation le receveur est loin d'être délivré de ses problèmes, et moins encore de ses médecins traitants. A la transplantation suit une période où le poumon transplanté se porte bien, mais par la suite de nouveaux problèmes se font jour sous forme de bronchiolite obliterans. Bien qu'inextricable au début, l'écheveau de causes de cette affection pernicieuse se laisse progressivement démêler. Cette évolution pourra engendrer des thérapies nouvelles susceptibles de prévenir et, si besoin est, de traiter la bronchiolite obliterans dans les poumons transplantés.

6.3 Deutsche Zusammenfassung

1. Die Entwicklung der Lungentransplantation

Durch die stürmische Entwicklung der Lungentransplantation in den letzten zehn Jahren, wird dieser Eingriff heutzutage mit viel Erfolg bei Patienten mit ernsten Lungenerkrankungen ausgeführt. Die meisten Empfänger von Lungentransplantaten können wieder ein normales Leben führen und viele von ihnen arbeiten sogar wieder. Das alles sieht sehr rosig aus, aber auch hier trügt der Schein. Obwohl die Erfolge im ersten Jahr nach der Lungentransplantation in den letzten 10 Jahren enorm verbeßert sind, verschlechtert sich bei einem großen Teil der Patienten die Funktion der transplantierten Lungen nach diesem ersten Jahr. Bei vielen Patienten wird die Lungenfunktion sogar so schlecht, daß diese Patienten sterben. Die 5-Jahres-Überlebenszeit, die in der Medizin für den Erfolg einer Behandlung verwendet wird, is nach einer Lungentransplantation kleiner als 50%, unabhängig van der Art der Lungentransplantation. Das heißt, das 5 jahre nach der Transplantation nur noch die Hälfte der operierten Patienten lebt. Es hat sich gezeigt, daß sich die Lungenfunktion bei beinahe allen verstorbenen Patienten durch eine Einengung der kleinen Luftwege auf Grund einer Entzûndungsreaktion verschlechtert hatte. Diese Entzûndung nennt man Bronchiolitis Obliterans. Die Ursache dieser Bronchiolitis Obliterans nach der Lungentransplantation ist leider unbekannt und deshalb schlecht zu behandeln. Bei Patienten, die kein Lungentransplantat erhalten haben, kann eine Bronchiolitis Obliterans nach einer Epithelbeschädigung durch virale Infektionen oder nach Einatmen toxischer Gase entstehen. Nach einer Epithelbeschädigung tritt in den meisten Fällen die selbe Entzündungsreaktion auf, ungeachtet verschiedener Ursachen. Warum Bronchiolitis Obliterans im Anschluß an eine Lungentransplantation so häufig vorkommt is unklar. Zur Zeit denkt man, daß diese Entzûndung durch eine Form der chronischen Immunreaktivität des Empfängers gegen die transplantierte Lunge verursacht wird, d.h. durch eine chronische Abstoßung. In der Klaßifikation der Internationalen Gesellschaft für Herz- und Lungtransplantation (International Society for Heart and Lung Transplantation) gehört die Bronchiolitis Obliterans dann auch zur Gruppe der chronischen Luftwegabstoßungen. Einen deutlichen Hinweis dafür, daß die Bronchiolitis Obliterans eine Abstoßungsreaktion is, findet mann darin, daß bereits vor dem Manifest werden der Bronchiolitis Obliterans, Zellen der Empfängers in der transplantierten Lungen gefunden werden, die gegen Gewebeantigene des Transplantates reagieren. Außerdem ist beschrieben, daß Bronchiolitis Obliterans haüfiger in Lungen vorkommt, die einige Male akut abgestoßen wurden. Möglicherweise spielen auch Virusinfektionen dabei eine Rolle. Diese kommen in transplantierten Lungen häufiger vor als in normalen Lungen. Der zu Grunde liegende Mechanismus is jedoch unbekannt. Die

Infektion mit dem Cytomegalovirus (CMV) scheint die wichtigste Ursache der Bronchiolitis Obliterans zu sein. Inzwischen weiß man, daß auch ander (virale) Infektionen der Lungen und Luftwege Bronchiolitis Obliterans verursachen können. Während einer scheinbar unschuldigen Luftweginfektion findet man eine Verschlechterung der Lungentransplantatsfunktion, von der sich die Lunge nicht wieder erholt. In Lungenbiopten sieht man erst eine aspezifische Bronchiolitis, die sich allmählig in eine Bronchiolitis Obliterans entwickelt. Obwohl man also weiß, daß Abstoßung und Infektion Ursachen einer Bronchiolitis Obliterans in Lungentransplantaten sein können, kann man auch den Verlauf davon für einen individuellen Patienten nicht vorhersagen. Deshalb ist es häufig unklar, was die wichtigste Ursache der Bronchiolitis Obliterans in der transplantierten Lunge ist.

Diese mangelnde Kenntnis über die Entwicklung einer Bronchiolitis Obliterans kann zu einer Sackgaße bei der Entwicklung der Lungtransplantation führen. Tierexperimentelle Studien können möglicherweise helfen, das Entstehen einer Bronchiolitis Obliterans beßer zu verstehen. Der große Vorteil tierexperimenteller Studien ist, daß man verschiedene Faktoren, von den man denkt, daß sie eine Bronchiolitis Obliterans in der transplantierten Lunge verursachen können, einzeln und gemeinsam untersuchen kan. Bei Versuchtieren, wie z.B. der Ratte kann man Inzuchtstämme züchten, deren Individuellen alle dieselben Gewebemerkmale haben. Wenn bei einer Transplantation Spender und Empfänger vom selben Stamm sind, wird keine Abstoßung der transplantierten Organs aftreten, weil die Gewebeantigene dieselbe sind: das nennt man eine syngen Transplantation. Wenn Spender und Empfänger jedoch von verschiedenen Stammen sind, also eine allogene Transplantation durchgeführt werd, wird Abstoßung auftreten. Wenn man immer dieselben Stämme verwendet, wird jedesmal eine gleiche Reaktionsart auftreten. So kann man u.a. untersuchen, ob nach einer Lungentransplantation Abstoßung zu einer Bronchiolitis Obliterans führen kan.

Vor etwa 15 Jahren haben Dr. K.W. Marck und Dr. Jm. Prop in Groningen ein Lungentransplantationsmodell entwickelt, bei dem nur die linke Lunge transplantiert wird. Vor allem die Arbeit von Prop hat unser Verständnis über den Mechanismus der akuten Lungenabstoßung enorm vergrößert. Mit diesem Lungentransplantationsmodell erwies sich, daß auch allogene Lungentransplantate längere Zeit überleben können bei gleichzeitiger immunosuppresiver Behandlung mit Zyclosporinen. Diese transplantierten Lungen werden nicht mehr akut abgestoßen, sondern funktionieren bis 1.5 Jahre nach der Transplantation gut. Für eine Laboratoriumsratte, die 2 bis 3 Jahre alt wird, ist das sehr lange. Mit diesem Modell haben wir die beim Menschen

häufigsten Ursachen einer Bronchiolitis Obliterans im Anschluß an eine Lungentransplantation untersucht: die chronische Abstoßung und die Virusinfektion.

2. Bronchiolitis Obliterans im Anschluß an die Lungen-transplantation bei der Ratte

Chronische Abstoßung

In der ersten Studie fanden wir, daß auch bei infektionsfrei gehaltenen Ratten chronische Abstoßung die Luftwege allogen transplantierter Lungen beschädigen kann. Wie bereits gesagt, werden allogen transplantierte Lungen unter Zyclosporinetherapie nicht mehr akut abgestoßen. Das bedeutet jedoch nicht, daß die transplantierten Lungen volkommen akzeptiert werden. In diesen allogen transplantierten Lungen entwickelt sich eine Form der Bronchiolitis, die der Bronchiolitis Obliterans beim Menschen ähnelt. Kurz nach der Transplantation wandern viele Lymphozyten aus der Blutbahn des Empfängers in die transplantierte Lunge. Diese Lymphozyten werden vor allem in der Umgebung der Blutgefäße und Luftwege der Lunge gefunden. Außerdem werden an einigen Stellen in der Lunge auf Epithelzellen Klasse-II-MHC-Gewebe-Antigene gebildet, was darauf hinweist, daß dort eine Entzündung stattfindet. Die Infiltrate in der Umgebung der Blutgefäße und Luftwege bilden sich in den anschließenden zwei Monaten zum größten Teil spontan zurück. Nach etwa 6 Monaten bleiben jedoch kleine Infiltrate in den großen Luftwegen bestehen, die auf eine chronische Abstoßung hinweisen. Diese Infiltrate setzen sich hauptsächlich aus T-Helfer-Zellen, dendritischen Zellen (das sind Zellen, die abweichende Zellen so an das Abwehrsystem präsentieren, daß selbiges aktiviert wird) und Klasse-II-MHC-Antigenen auf Epithelzellen zusammen. In diesen Infiltraten ist das Epithel beschädigt und kleine Stücke Narbengewebe wachsen in das Lumen der Luftwege. Dieser Prozeß bleibt mindestens bis 1.5 Jahre nach der Transplantation stabil oder is langsam progreßiv und beschränkt sich in dieser Periode auf die großen Luftwege. Da syngen transplantierte Lungen nach 1.5 Jahren vollkommen normale Luftwege haben, sind diese Luftwegveränderungen wirklich eine chronische Abstoßungsreaktion und nicht Folgen der Transplantation. Diese Experimente zeigen deutlich, daß chronische Abstoßung ohne infektion Abweichungen in transplantierten Rattenlungen verursachen. Die bei Bronchiolitis Obliterans, die wir beim Menschen sehen, is jedoch viel ernster und kommt hauptsächlich in den kleinen luftwegen vor. Das weist darauf hin, daß bei der Entwicklung der ernsten Bronchiolitis Obliterans, bei Lungen-transplantationspatienten möglicherweise ein weiterer Faktor eine Rolle spielt.

Da Virusinfektionen neben der chronischen Abstoßungsreaktion immer wieder als mögliche Ursache der Bronchiolitis Obliterans genannt werden, haben wir in den folgenden Experimenten den Einfluß virale Infektionen auf allogen transplantierte Lungen untersucht.

Virusinfektionen

Virale Luftweginfektionen verschlimmern die bereits wahrgenommene Form der Bronchiolitis Obliterans (Kapitel 2.2). Das zeigten wir durch Infizierung dieser Lungen mit dem Sendai Luftwegvirus (Parainfluenza Typ I). In normalen Lungen verursacht das Sendai Virus eine leichte Bronchiolitis, die innerhalb von 3 Wochen volkommen geheilt ist. In der allogen Transplantierten Lungen entwickelt sich jedoch innerhalb einer Woche eine ernste Bronchiolitis, mit stark beschädigtem Epithel und einer ernsthaften Entzündung. Sowohl das Epithel der großen als auch der kleinen Luftwege is ernsthaft beschädigt. Die Bronchiolitis nimmt bis zu 3 Wochen nach der Infektion zu und bleibt während der gesamten Dauer des Experimentes, 8 Wochen, bestehen. Als Folge dieser starken Entzündung entwickelt sich eine Bronchiolitis Obliterans mit einer deutlichen Einengung vor allem der kleinen Luftwege so wie sie auch bei Patienten nach einer Lungentransplantation gesehen wird.

Auch bei diesen Experimenten reagieren syngen transplantierte Lungen genauso wie normale, nicht transplantierte Lungen. Der Verlauf der Infektion is genau derselbe wie in normalen Lungen. Diese Studie is der erste experimentelle Beweis, daß eine ernsthafte Bronchiolitis Obliterans in Lungentransplantaten durch eine Kombination von chronischer Abstoßung und viraler Infektion verursacht wird. Aber was geht eigentlich schief, bei gleichzeitigem Auftreten chronischer Abstoßung und Infektion? Um darüber mehr zu erfahren, haben wir in der folgenden Studie die Entzündungsreaktion in der infizierten transplantierten Lunge genauer untersucht.

Entzündungsreaktionen

Die ernste Bronchiolitis Obliterans im Anschluß an eine Virusinfektion in der allogen transplantierten Lunge wird durch eine abnormal starke Entzündungsreaktion kurz nach der Infektion verursacht (Kapitel 2.3). Im Gegensatz zur Situation in der syngen transplantierten Lunge kommen während dieser starken Entzündungsreaktion sehr viel aktivierte Makrophagen und neutrophile Granulozyten vor. In erster Linie ist diese starke Entzündungsreaktion dafür verantwortlich, daß das Epithel angegriffen wird und die Luftwege im Anschluß daran vernarben. Außerdem reagieren die zytotoxischen T-Lymphozyten, die eine wichtige Rolle in der Abwehr von

Virusinfektionen spielen, in allogen transplantierten Lungen viel träger als in syngen transplantierten und in nicht transplantierten, normalen Lungen. In allogen transplantierten Lungen dauert es etwa 3 Wochen bevor sie in normaler Konzentration vorkommen. Zu diesem Zeitpunkt sind die Luftwege schon durch die Infektion beschädigt. Außerdem wandern in der allogen transplantierten Lunge, im Gegensatz zur syngen transplantierten Lunge, kaum B-lymphozyten, die Zellen die Antistoffen bilden, zu den Luftwegen. Warscheinlich ist die Virusabwehr in allogen transplantierten Lungen gestört. Das könnte auch erklären, warum in transplantierten Lungen beim Menschen soviel Infektionen vorkommen. Weil es noch sehr wenig Kenntniße über die Funktionsweise des Abwehrsystems in transplantierten Lungen gibt, konzentrieren wir uns im zweiten Teil dieser Arbeit darauf.

3. Gestörte Abwehr gegen Infektionen in transplantierten Lungen

Während jedes Atemzuges erreichen allerlei Teilchen die Lunge, Staub, Viren und Bakterien. Um vorzubeugen, daß wir diesen Teilchen erliegen, sind unsere Lungen mit einem sehr ausgebreiteten Abwehrsystem ausgestattet. Beim gesunden Menschen macht dieses Abwehrsystem die eingeatmeten Pathogene unschädlich. Diese Abwehrmechanismen variieren vom unspezifischen Hustreflex bis zu komplexen immunologischen Reaktionen, wobei spezifische Antikörper gegen eingeatmete Antigene gebildet werden (Kapitel 3.1). Diese Antikörperproduktion erfolgt im lymphatischen Gewebe, das innerhalb und außerhalb der Lung vorkommt. Hauptsächlich die Antikörper, die lokal, innerhalb der Lungen im BALT (bronchus-aßociated-lymphoid-tißue) gebildet werden sind sehr wichtig als erste Verteidigung gegen Viren un Bakterien. Außerdem is die Antikörperproduktion in den Lymphknoten in der Nähe der Lunge wichtig für den Schutz der Lung und des übrigen Körpers.

In diesem Kapitel beschreiben wir die Studie über die Antikörperproduktion in Blut und Lokal in der Lunge gegen zwei in die transplantierte Lunge gebrachte antigene: Schafserythrocyten und das Sendai Virus.

Antikörperproduktion gegen Schafserythrozyten

Die Antikörperproduktion im blut gegen in die Lunge gespritzte Schafserythrozyten erweist sich nach der Lungentransplantation als gestört. Auffallend ist, daß das allerdings nur kurz nach der Transplantation der Fall ist (Kapitel 3.2). Ein Monat nach der Transplantation kann eine normale Antikörperproduktion in allogen transplantierte Ratten ausgelöst werden. Diese zeitliche Störung der Antikörperproduktion wird durch das Durchschneiden der

Lymphgefäße zu den Lymphknoten der Lunge während der Transplantation verursacht.

In Experimenten, in den nur die Lymphbanen durchgeschnitten werden, ohne die Lunge zu transplantieren, regenerieren die Lymphgefäße sich innerhalb eine Woche. Gleichzeitig kommt die Antikörperproduktion wieder auf Gang (Kapitel 3.3). Nach zwei wochen hat sich die Antikörperproduktion wieder volkommen normalisiert. Glücklicherweise bilden sich auch nach allogener Lungentransplantation wieder Lymphbanen. Diese Resultate sind ein Hinweis dafür, daß die Lungen in den ersten beiden Wochen nach Lungentransplantation besonders empfindlich für infektionen sind, weil die Lymphgefäße durchgetrennt sind. Deshalb is es wichtig, in dieser ersten, empfindlichen Periode nach der Transplantation besonders auf entstehende Infektionen zu achten und stark vorbeugende Maßnahmen einzuhalten während diagnostischer Eingriffe, wie dem Bioptieren der Lunge, von dem man weiß, daß es ein wichtiger Infektionsbrunnen sien kann.

Antikörperproduktion gegen das Sendai Virus

Antikörperproduktion gegen das Sendai Virus, systemisch im Blut und lokal im BALT, ist nach allogener Lungentransplantation gestört (Kapitel 3.4). Im gegensatz zur Reaktion auf Schafserythrozyten ist diese Störung bleibender Art. Am auffälligsten ist die geringe Anzahl Antikörper produzierender Zellen im BALT nach der Infektion der allogenen Lungentransplantate. Durch die viel geringere Antikörperproduktion kommt das Virus in diesen Lungen viel länger vor. Im gegensatz dazu ist in syngen transplantierten un nicht transplantierten Lungen, in denen die Zahl Antikörper produzierender Zellen nach Infektion stark zunimmt, das Virus lediglich einige Tage nachweisbar. Warscheinlich wird das BALT durch die Allotransplantation so beschädigt, daß es nach einer Infektion nicht mehr normal funktionieren kann.

In sich anschließenden Experimenten haben wir in Lungentransplantaten 3 wichtige Voraußetzungen für eine normale BALT-Funktion untersucht: die Struktur, die Antigenaufnahme aus den Luftwegen und die Lymphozytenmigration aus dem Blut in das BALT (Kapitel 3.5). Es zeigt sich, daß alle drei Voraußetzungen des BALT in der allogen transplantierten Lunge ernsthaft gestört sind. Das BALT is viel kleiner, hat viel weniger Lymphozyten als normalerweise und is zum großen Teil durch Narbengewebe ersetzt. Außerdem ist die Aufname der Teilchen aus den Luftwegen gestört und die Lymphozytenmigration in das BALT stark verringert. Diese starke Beschädigung erklärt das Ausbleiben der Antikörperproduktion nach einer Infektion in diesen

Lungen. Höchstwahrscheinlich wird das BALT durch Abstoßung beschädigt, da es in syngen transplantierten Lungen vollkommen normal ist. Prop hatte in einer früheren Studie gezeigt, daß das BALT das erste Zielobjekt einer unbehandelten akuten Abstoßung ist. Leider is das warscheinlich auch der Fall, wenn die akute Abstoßung mit Zyklosporinen behandelt wird. Im Kapitel 5 wird weiter darauf eingegangen, welche vorbeugenden Maßnahmen die Beschädigung des BALT in allogen transplantierten Lungen verhindern können.

4. Die Immunreaktion nach Lungentransplantation beim Menschen

Aus oben Genanntem zeigt sich, daß Bronchiolitis Obliterans nach der Lungentransplantation in der Ratte durch das Entgleisen von Entzündungsreaktionen und Abwehrmechanismen der transplantierten Lunge nach Abstoßung und Infektion entsteht. Obwohl Ergebniße tierexperimenteller Studien nicht ohne weiteres auf die klinische Situation angewendet werden dürfen, rechnen wir damit, daß Bronchiolitis Obliterans nach Lungentransplantation beim Menschen durch die selben entgleisten Reaktionen entsteht wie bei der Ratte (Kapitel 4.1). Eine frühzeitige Erkennung dieser Reaktionen könnte eine schnelle Behandlung ermöglichen und dadurch vielleicht das Entstehen der Bronchiolitis Obliterans verhindern. Entzündungsreaktionen und Abwehrmechanismen nach der Abstoßung und Infektion erfolgen im Lungengewebe. Deshalb können sie am besten in einem Biopt aus der transplantierten Lunge entdeckt werden. Am sichersten und ungefährlich gewinnt man Gewebe durch ein transbronchiales Biopt. Hierbei nimmt man kleine Stückchen Gewebe aus der transplantierten Lunge, in denen idealerweise Luftwegepithel, Blutgefäße, und anderes Lungengewebe vorkommt. Aus der Praxis weiß man, daß in diesen Biopten nicht immer genug Gewebe vorkommt. Vor allem Luftwegepithel fehlt häufig. Dadurch ist es schwierig oder unmöglich, einen guten Eindruck von der Beschädigung des Lungengewebes zu bekommen. Der spezifische Nachweis verschiedener Zellen, die an Entzündungs- und Abwehrreaktionen teilnehmen, wie die weiter oben genannten Makrophagen und Lymphozyten, kann jedoch ein Hinweis auf eine mögliche Beschädigung der Luftwege und des Lungengewebes sein.

In einer klinischen Studie haben wir das Vorkommen dieser Zellen während früher Phasen der akuten und chronischen Lungenabstoßung nach einer Herz-Lungen-Transplantation untersucht. Wir fanden, daß es für ein beßeres Verständnis der Entzündungs- und Abwehrreaktionen in Lungentransplantaten spezifisische Zellen zufinden, sinnvoll sein kann, auch wenn nur wenig Gewebe in den Biopten vorhanden ist (Kapitel 4.2). In der frühen Phase der akuten und

chronischen Abstoßung fanden wir infiltrierenden Zellen in der Umgebung der Blutgefäße, die den infiltrierenden Zellen in den Luftwegen glichen. Die Entzündungsreaktion während der akuten Abstoßung was stärker als die während der chronische Abstoßung, mit einer auffallend großen Anzahl Makrophagen.

Diese Studie beweist, daß das Vorkommen von spezifischen Zellen in Lungenbiopten uns mehr information über die immunologischen Prozeße in transplantierten Lungen gibt, selbst wenn nur wenig Gewebe vorhanden ist. Wichtig ist im Zusammenhang mit den Ergebnißen der vorangegangenen Kapitel auch, daß sich beim Menschen nach der Lungentransplantation tatsächlich die selben Reaktionen abzuspielen scheineStudien gemeinsam helfen, die Mechanismen, die zu einer Bronchiolitis Obliterans nach der lungentransplantation führen, zu verstehen.

5. Ursachen der Bronchiolitis Obliterans in Lungentransplantaten

Zusammenfaßend kommen wir zu folgender Hypothese über das Entstehen der Bronchiolitis Obliterans nach Lungentransplantation: chronische Abstoßung verursacht nach der Transplantation eine leichte Beschädigung der Luftwege der transplantierten Lunge. Wichtiger ist jedoch, daß dieselbe Abstoßungsreaktion das lokale Abwehrsystem der transplantierten Lunge so beschädigt, das dieses nicht mehr normal auf Infektionen reagieren kann. Wenn die Lunge dann durch eine Luftwegvirus infektiert werd, entsteht eine abnormal starke und lange Bronchiolitis mit starker Vernarbung der kleinen Luftwege: das Bild der Bronchiolitis Obliterans. Andere Faktoren, wie z.B. eine Ischämie, können diesen Prozeß noch verstärken.

Die Prävention der Bronchiolitis Obliterans muß auf zwei Gebieten erfolgen. Zum ersten, eine agreßive Unterdrückung der Abstoßung in einem frühen Stadium nach der Transplantation, um das lokal Abwehrsystem der Lung zu erhalten. Und zweitens, das Unterdrücken der abnormal starken Entzündungsreaktionen in der transplantierten Lunge nach Infektionen. Offensichtlich reichen die heutigen Behandlungsmethoden dafür noch nicht aus, obwohl immer mit mehreren Immunosuppressiva behandelt wird, einschließlich hoher und permanent gegebener Kortikosteroide. Es ist jedoch vorstellbar, daß diese Medikamente, aus Vorsicht vor dem Risiko ernsthafter Nebenwirkungen, häufig zu spät und doch in einer zu niedrigen Dosierung gegeben werden. Außerdem könnte die notwendige lokale Konzentration ungenügend hoch sein, um die starke Entzündungsreaktion zu unterdrücken. Möglicherweise ist das Problem zu lösen, indem man die Medikamente lokal in die transplantierte Lunge zudient, wie auch bei der Asthmabehandlung stets mehr Medikamente via Inhalation eingenommen werden. Das könnte auch nach der

Lungentransplantation eine gute Alternative sein, angesichts dessen, daß die Reaktionen, die zu einer Bronchiolitis Obliterans führen, hauptsächlich lokal in der Umgebung der Luftwege vorkommen und so leicht durch inhalierte Stoffe erreichbar sind. Sowohl Abstoßung als auch Entzündung können auf diese Art und Weise behandelt werden.

Eine andere, noch experimentelle Behandlung des Lungentransplantates, um eine Abstoßung vor allem des BALT zu verhindern, ist die Bestrahlung der Spenderlunge vor Entnahme der Lunge aus dem Spender, wodurch die stark immunogenen Lymphozyten im BALT der zu transplantierenden Lunge verschwinden. Die Struktur des BALT könnte in diesen bestrahlten Lungen beßer erhalten bleiben und sogar eine Wiederbesiedlung mit Lymphozyten des Empfängers ermöglichen. Möglicherweise könnte dadurch die Funktion des BALT wieder hergestellt werden und eine normale Abwehr gegen Infektionen entstehen.

Schluß

Aus oben Genanntem zeigt sich, daß obwohl die Lungentransplantation heutzutage relative einfach ausgeführt ist, noch lang nicht das letzte Word über die Transplantation gesprochen ist. Der operierte patient is noch lange nicht von seinen Problemen, geschweige van seinen Ärtzten erlöst. Eine Zeit lang geht es mit der transplantierten Lunge gut, aber dann entstehen wieder neue Problem, z.B. durch eine Bronchiolitis Obliterans. Obwohl die Summe der Ursachen anfänglich untrennbar erschien, gelingt es langsam die einzelnen Ursachen dieser tückischen Kranktheit zu erkenne. Dadurch können zweckmäßige Therapien entwickelt werden, die eine Bronchiolitis Obliterans in transplantierten Lungen verhindern bzw. behandeln.

6.4 Súhrn slovensky

1 Vyvoj transplantácie plúc

V dôsledku búrlivého rozvoja transplantácie plúc za poslednych desat´ rokov, dá sa v súcastnosti u pacientov s váznymi chorobami plúc vykonat´ tento zákrok s velkym úspechom. Väcsina príjemcov plúcnych transplantátov môze opät´ viest´ normálny zivot, ba dokonca mnohí sa vrácajú spät´ k svojej práci. Toto vsetko s zdá byt´ rúzové, no ukázalo sa, ze i tu pekny lesk klame. V prvom roku po transplantácii plúc sa za obdobie poslednych 10 rokov vysledky podstatne zlepsili. Po tomto prvom roku sa vsak znizuje funkcia transplantoványch plúc u velkej casti pacientov. U mnohych pacientov sa znizuje natolko, ze to je pricínou smrti. V medicíne pouzívané "prezitie piatich rokov" na vyjadrenie úspechu liecby, je po transplantácii plúc menej ako 50%, odhliadnúc od typu transplantácie. To znamená, ze 5 rokov po transplantácii plúc uz iba menej ako polovica transplantovanych pacientov preziva. Ukazuje sa, ze u vsetkych pacientov, ktori zomreli, klesá funkcia plúc, ako následok obstrukcie dolnych ciest dychacích v plúcach, zaprícinenej zápalovou reakciou. Toto ochorenie nezyveme bronchiolitis obliterans. Prícina bronchiolitis obliterans po transplantácii plúc zial nie je jasná a preto je aj tazko liecitelná.

U pacientov, ktorí nie sú transplantovaní, môze bronchiolitis obliterans vzniknút´ po poskodení epitelu vírusovymi infekciami alebo vdychnutím toxickych plynov. Zápalová reakcia, ktorá po tomto poskodení epitelu vznikne v dolnych dychacích, cestách je vo väcsine prípadov rovnaká, hoci príciny sú rozne. Nie je este jasné, co je prícinou toho, ze po transplantácii plúc dochádza k tak castemu vyskytu bronchiolitis obliterans. V súcasnosti sa predpokladá, ze zápal v dychacích cestách je zaprícineny akousi formou imunoreaktivity príjemcu transplantovanych plúc tzv chronická rejekcia, ale tiez (vírusovymi) infekciami plúc, ktoré sa po transplantácii velmi casto vyskytujú. V klasifikácii spolocnosti "International Society for Heart and Lung Transplantation" je bronchiolitis obliterans zaradená do skupiny "chronic airway rejection". Bunky príjemcu, ktore sa správajú reaktívne proti tkánivovému antigénu transplantátu, mozno nájst´ v transplantovanych plúcach skôr, ako sa bronchiolitis obliterans manifestuje, co jasne poukazuje na súvis brochiolitis obliterans s rejekciou. Je tiez popísané, ze bronchiolitis obliterans sa castejsie vyskytuje v plúcach, ktoré uz prekonali viaceré "epizódy" chronickej rejeckcie.

Je opätovne uvádzaná súvislost´ infekcie s bronchiolitis obliterans, ale jej mechanizmus nie je jasny. Predovsetkym infekcia spôsobena cytomegalovírusom (CMV) sa uvádza ako najhlavnejsia prícina bronchiolitis obliterans, avsak medzitym sa uz ukazuje stále jasnejsie, ze aj iné (vírosové) infekcie plúc a dychacích ciest môzu zaprícinit´ bronchiolitis obliterans. Pocas zdánlivo nevinnej infekcie dychacích ciest pozorujeme pokles funkcie plúcného transplantátu, ktorá

sa uz potom neobnovuje. Vó vzorkách z materiálu získného biopsiou plúc pozorujeme najprv nespecifickú bronchiolitis, ktorá sa potom pozvolna rozvinie do jasnej specifickej bronchiolitis obliterans. Hoci teda rejekcia a infekcia môzu byt´ prícinami bronchiolitis obliterans. V plúcnych transplantátoch, nie je mozné ich vyskyt a priebeh u jednotlivych pacientov predpovedat´. Preto je casto nejasné, co je najhlavnejsou prícinou bronchiolitis obliterans v transplantovanych plúcach.

Pre tento nedostatok poznatkov o vzniku bronchiolitis obliterans hrozí nastat´ akási bezvychodísková situacia vo vyvoji transplantácie plúc u ludí. Vyskum na pokusnych zvieratách by mozno mohol pokystnút´ poznatky o vzniku bronchiolitis obliterans po transplantácii plúc. Velkou vyhodou vyskumu na pokusnych zvieratách je to, ze rôzne faktory, o ktorych sa predpokladá, ze zaprícinujú bronchiolitis obliterans v transplantovanych plúcach, môzu byt´ skúmané jednotlivo i v kombinácii. U pokusnych zvierat, na pr. u krys, mozno krízením získat´ kmene, kde vsetci jednotlivci majú tie isté - rovnaké znaky tkániva. Ak pri transplantácii darca a príjemca sú z toho istého kmena, nenastane rejekcia transplantátu, preto ze ich tkanivové antigény sú rovnaké - syngénna transplantácia. Avsak ak darca a príjemca sú z rôznych kmenov - alogénna transplantácia- nastane rejekcia. Ak sa vzdy pouzijú tie isté kmene, vieme vzdy isto, ze nastane ten isty druh reakcie. Tak môzeme, okrem iného, preskúmat´ faktor rejekcie, ci tento má vplyv na vznik patologickych zmien v smysle ako bronchiolitis obliterans, po transplantácii plúc.

Asi pred 15 rokmi vyvinuli dr. K.W. Marck a dr. Jm. Prop model transplantácie plúc pre krysy, pri ktorom sa transplantovali iba lavé plúca. Menovite práca Propa zvãcsila nase poznatky o mechanizme akútnej rejekcie plúc. S tymto transplantacnym modelom plúc, po osetrení cyclosporinom, sa ukázalo mozné docielit´ dlhodobé prezitie alogénne transplantovanych plúc. Tieto transplantatované plúca neboli uz akútne odmietnuté, ale mohli dobre pracovat´ az do jeden a pol roka, ca je velmi dlho pre laboratórnu krysu, ktora sa vãcsinou dozíva iba 2-3 rokov. Naj pravdepodovnejsie príciny vzniku bronchiolitis obliterans po transplantácii pluc u cloveka sme s tymto modelom zistili: chronickú rejekciu a vírusové infekcie.

2 Bronchiolitis obliterans po transplantácii plúc u krysy

Chronická rejekcia

V prvom vyskume (Kapitola 2.1) sme zistili, ze chronická rejekcia u krys, drzanych bez nebezpecia nákazy, môze skutocne spôsobit poskodenie dychcích

6.4 Súhrn slovensky

ciest alogénne transplantovanych plúc. Ako uz bolo povedané u alogénne transplantovanych krysích plúc, liecenych cyclosporínom,nedôjde k rejekcii. To vsak neznamená,ze transplantované plúca budú úplne prijaté. Ukázalo sa totiz, ze tu vznikala akási forma bronchiolitis, ktorá sa podobá bronchiolitis obliterans u cloveka. Skoro po transplantácii putuje vela lymfocytov z krvného obehu príjemcu do transplantovanych plúc. Tieto lymfocyti boli prítomné predovsetkym okolo ciev a v dychcích cestách plúc. Pri tom sa na niektorych miestach plúc v epitelovych bunkách zistil vyrazny prejav tkánivového antigénu tzv. triedy II (MHC). To je znak toho, ze v okolí tychto epitelovych buniek prebieha zápalovy proces. Infiltráty okolo krvnych ciev a hornych ciest dychacích v nasledujúcich 2 mesiacoch v preváznej miere spontánne vymizli. Avsak asi po 6 mesiacoch ostali v bunkách dolnych ciest dychacích drobné infiltráty, ktoré poukazujú na chronickú rejekciu: najmä pomocného typu T lymfocyty, nahromadenie (príjemcovych) dendritickych buniek (to sú bunky, ktoré odlisné a telu nevlastné bunky kontaktujú s obrannym systémom, aby tento mohol byt aktivovany) a tkaninovy antigén triedy ll na epiteli. Epitel bol pri tychto infiltrátoch lokálne poskodeny kúsky zjavneneho tkaniva rástli v otvore dychacích ciest. Potom doslo k stabilite procesu, alebo len pomalému zhorseniu najmenej do jeden a pól roka po transplantácii, a pocas tohoto casu ostal proces obmedzeny na horné cesty dychacie. Tieto zmeny dychacích ciest sú zaprícinené chronickou rejekciou a nie následkami transplantácie, co je potvrdené faktom, ze syngénne transplantované plúca mali jeden a pól roka po transplantácii úplne normálne dychacie cesty. Tieto experimenty velmi jednoznacne poukazujú na to, ze chronická rejekcia bez infekcie zaprícinuje zmeny v transplantovanych krysích plúcach. Kedze bronchiolitis obliterans , tak ako ju vydíme u ludí, je ovela váznejsia a predovsetkym lokalizovaná v dolnych cestách dychacích , poukazujú tieto vysledky na to, ze mozno existuje aj druhy faktor, ktory spolu s chronickou rejekciou vypudzovaním zaprícinuje váznu formu bronchiolitis obliterans u pacientov s transplantáciou plúc. Kedze vírusové infekcie vedla chronickej rejekcie sú stále spomínané ako mozná prícina bronchiolitis obliterans, preskúmali v následujúcich experimentoch úcinok vírusovej infekcie v alogénne transplantovanych plúcach.

Vírusové infekcie

Preukázalo sa, ze vírusova infekcia dychacích ciest silno zhorsila uz predtym zistenú lahkú formu bronchiolitis (Kapitola 2.2). To sme preukázali tym, ze sme plúca s chronickou rejekciou nakazili Sendai vírusom dychcích ciest (Parainfluenza typu l). Sendai vírus zaprícinuje v normálnych plúcach lahkú formu bronchiolitis, ktorá vsak je vzdy po troch tyzdnoch úplne vyliecená.

V transplantovanych plúcach s chronickou rejekciou sa v priebehu jedného tyzdna vyvinula vyrazná bronchiolitis, so silne poskodenym epitelom a prudkym zápalom. Epitel bol vyrazne poskodeny u dolnych ako aj hornych dychacích ciest. Priebeh ochorenia gradoval do 21 dní po infekcii, a ochorenie ostalo pocas celej periody experimentu, t.j. 8 tyzdnov, prítomné. Ako následok tejto prudkej zápalovej reakcie vznikla bronchiolitis obliterans, ktorej obraz bol identicky s obrazom u pacientov po transplantácii plúc, so zjavnymi obliteraciami najme dolnych ciest dychacích.

Aj pri tychto experimentoch sa ukázalo, ze syngénne transplantované plúca reagovali tym istym spôsobom ako zdravé netransplantované plúca, pretoze priebeh infekcie bol presne identicky s jej priebehom v normálnych plúcach. Tento vyskum je prvym experimentálnym dôkazom toho, ze silná (vyrazná) bronchiolitis obliterans v plúcnych transplantátoch byva zaprícinená kombináciou chronickej rejekcie a vírusovej infekcie. Co sa vsak nedarí pocas súcasného vyskytu chronickej rejekcie a vírusovej infekcie? So snahou získat viac poznatkou, zaoberali sme sa v nasledujúcom vyskume ovela podrobnejsie skúmaním zápalovych reakcií v transplantovanych plúcach s infekciou.

Zápalové reakcie

Silná (vyrazná) bronchiolitis obliterans po vírusovej infekcii v alogénne transplantovanych plúcach sa zdá byt zaprícinená abnormálne silnou zápalovou reakciou spôsobenou v cas (bezprosredne) po infekcii (Kapitola 2.3). Pocas tejto prudkej zápalovej reakcie bola zistená velká prítomnost aktivovanych makrofágov a neutrofilnych granulocytov, ktoré v syngénne transplantovanych plúcach sa vyskytovali po infekcii v ovela mensej miere. Je to predovsetkym táto prudká zápalová reakcia v alogénne transplantovanych plúcach, ktorá napadá epitel, vysledkom coho je silné zjavenie dychcích ciest. Dalej pozorujeme, ze bunky, ktoré hrajú vyznamnú úlohu v obrane proti vírusovym infekciám - citotoxické T lymfocyty - reagujú v tychto plúcach ovela spomalenejsie, ako v syngénne transplantovanych a netransplantovanych plúcach. Trvalo asi 3 tyzdne, kym tieto bunky v alogénne transplantovanych plúcach boli opät prítomné v normálnom pocte. Tiez sa ukázalo, ze skoro ziadne B lymfocyty - bunky vytvárajúce protilátky -, neputovali do dychacích ciest, na rozdiel od syngénne transplantovanych plúc, kde tomu tak bolo. Ukazuje sa teda, ze obrana proti vírusom je v alogénne transplantovanych plúcach porusená. Toto by mohlo byt vysvetlenie prícin vyskytu velkého poctu infekcií v transplantovanych plúcach u ludí. Kedze je málo známeho o fungovaní obranného systému v transplantovanych

plúcach, skúmali sme v druhej casti násho vyskumu, ako to je s obrannym systémom po transplantácii plúc.

3 Porusená obrana proti infekciám v transplantovanych plúcach.
Pocas kazdého nadychnutia vniknú do plúc rôzne drobné ciastocky, pocínajúc prachom az po vírusi a baktérie. Nase plúca sú vyzbrojené rozsírenym obrannym systémom, aby nepodlahli tejto invázii patogénov. U zdravého cloveka tento obranny systém vdychnuté patogény zneskodí. Tieto obranné systémy sú rôzne; od nespecifického reflexu kasla po komplexné imunologické reakcie, pri ktorych sa tvoria protilátky, specificky zamerané na vdychnuty antigén (Kapitola 3.1). Táto tvorba protilátok sa deje v lymfoidnej tkáni, ktorá sa nachádza na rôznych miestách vnútri i pomimo plúc. Menovite protilátky, ktoré sa lokálne tvoria v lymfoidnej tkáni plúc BALT (bronchus associated lymphoid tissue), sú velmi dôlezité ako prvá obranná línia proti vírusom a baktériam. Popritom je tvorba protilátok v lymfatyckych uzlinách v blízkosti plúc, dôlezitá pre ochranu plúc a ostatného tela.

V tejto kapitole popisujeme vyskum tvorby protilátok v krvi a lokálne v plúcach proti dvom antigénom, zanesenym do plúc: ovcie erytrocyty (SRBC) a Sendai vírus.

Reakcia protilátok proti ovcím erytrocytom

Tvorba protilátok v krvi proti ovcím erytrocytom vstreknutím do plúc sa po transplantácii plúc ukazuje byt porusená. Bolo vsak nápadné, ze toto bolo iba krátko po transplantácii (Kapitola 3.2). Mesiac po transplantácii bolo mozné vyvolat normálnu tvorbu protilátok u alogénne transplantovanych krys. Ukázalo sa, ze táto, casovo abmedzená porucha tvorby protilátok, je následkom prerezania lymfatyckych ciev vedúcich do lymfatickych uzlín plúc, pocas transplantácie.

V experimentoch, pri ktorych sme,bez transplantácie plúc, iba prerezali lymfatické cievy, sa ukázalo, ze asi po jednom tyzdni sa tieto spontánne obnovili a paralelne s tym zacala aj tvorba protilátok (Kapitola 3.3).
Po 2 tyzdnoch bola tvorba protilátok opät úplne normalizovaná. Na stastie sa ukázalo, ze tieto lymfatycké cievy sa obnovia aj v alogénne transplantovanych plúcach.

Tieto vysledky poukazujú na to, ze v prvych dvoch tyzdnoch po transplantácii sú plúca citlivé na infekcie, pretoze lymfatycké cievy prerezané. Preto je dôlezité v tejto priode skoro po transplantácii, venovat mimoriadnu pozornost moznosti vyskytu infekcií. V tejto zranitelnej periode sa nám vidí rozumné vykonat silné preventívne opatrenia pocas rôznych diagnostickych zákrokov, ako je napr. biopsia plúc, ktoré môzu byt, ako je známe, váznym zdrojom infekcie.

Reakcia protilátok proti Sendaivírusu

Aj systemická tvorba protilátok v krvi, ako aj lokálna v BALT proti Sendaivírusu, je po alogénnej transplantácii plúc zmensená (Kapitola 3.4). Velky rozdiel proti ovcím erytrocytom bol vsak v tom, ze táto porucha bola trvalá. Najnápadnejsí bol maly pocet buniek tvoriacích protilátky v BALT po infekcii v tychto alogénnych plúcnych transplantátoch. Pre túto vyrazne znízenú tvorbu protilátok bol vírus v tychto plúcach ovela dlhsie prítomny. Toto bolo v ostrom protiklade so syngénne transplantovanymi a netransplantovanymi plúcami, u ktorych po infekcii vyrazne stúpol pocet buniek tvoriacich protilátky a vírus bol v plúcach dokazatelny iba niekolko dní. Javí sa teda, ze BALT sa alotransplantáciou poskodí, preto potom nemôze pri infekcii normálne fungovat.

Z toho dôvodu sme v následnych experimentoch v plúcnych transplantátoch preskúmali 3 vyznamné podmienky pre normálne fungovanie BALT: struktúru, prijímanie antigénov z dychcích ciest a putovanie lymfocytov z krvného obehu do BALT (Kapitola 3.5). Ukázalo sa, ze BALT v alogénne transplantovanych plúcach pri vsetkych troch podmienkach bol vázne napadnuty. BALT bol ovela mensí, obsahoval ovela menej lymfocytov ako normálne a bol z velkej casti nahradeny zjavnenym tkanivom. Dalej sa ukázalo, ze bol poruseny príjem ciastociek z dychcích ciest a putovanie lymfocytov do BALT sa vyrazne zmensilo. Toto silné poskodenie vysvetluje, ze v tychto plúcach nenastane po infekcii tvorba protilátok. Je nanajvys pravdepodobné, ze BALT je poskodeny rejekciou, pretoze BALT v syngénne transplantovanych plúcach bol neposkodeny. Aj predchádzajúci vyskum Propa ukázal, ze BALT je prvym tercom neliecitelnej akútnej rejekcie a ukazuje sa, ze zial toto tiez platí, aj ked je akútna rejekcia liecená cyclosporínom. V Kapitole 5 pojednávame o moznych preventívnych opatreniach, ktoré by mohli predíst poskodeniu BALT v alogénne transplantovanych plúcach.

4 Imunoreakcia po transplantácii plúc u cloveka

Z vyssie uvedeného vyplyva, ze bronchiolitis obliterans po transplantácii plúc u krysy vzniká vykolajením zápalovych a obrannych reakcií po rejekcii a infekcii v transplantovanych plúcach. Hoci vysledky vyskumu na pokusnych zvieratách nemozno bez vsetkého prenásat do klinickej situácie, vychádzame z toho, ze bronchiolitis obliterans po transplantácii u cloveka vzniká tymi istymi porusenymi reakciami ako u krysy (Kapitola 4.1). Vcasné zistenie tychto reakcií by umoznilo skoré liecenie a tym aj prevenciu bronchiolitis obliterans. Zápalové a obranné reakcie, po rejekcii a infekcii, sa odohrávajú v plúcnom tkanive a preto tam môzu byt najlepsie zistené biopsiou tkaniva získanou z transplantovanych plúc. To mozno vykonat spolahlivym a bezpecnym spôsobom, s tzv transbronchiálnou biopsiou. Pri tom sa z transplantovanych plúc odoberú malé ciastocky tkaniva, v ktorom, ak je to v poriadku, sú kúsky epitelu dychcích ciest, krvné cievocky a iné

plúcne tkanivo. V praxi sa vsak ukazuje, ze v tychto vzorkách nie vzdy je dostatok tkaniva, pricom predovsetkym casto chyba epitel dychcích ciest.Tymto je tazké alebo nemozné získat správny obraz o poskodení plúcneho tkaniva. Avsak specifické zistenie rozlicnych druhov buniek, zúcastnenych na zápalovych a obrannych reakciách, ako predtym menované makrofágy a lymfocyty, by mohlo poukazovat na moznost poskodenia dychcích ciest a plúcneho tkaniva.

V klinickej stúdii sme zistovali prítomnost tychto buniek v pociatocnej fáze akútnej a chronickej rejekcii plúc po transplantácii srdce - plúca. Zistili sme, ze detekcia specifickych buniek skutocne môze byt velmi úcinná z dôvodu lepsieho nazretia do zápalovych a obrannych reakcií v plúcnych transplantátoch, al ked je v biopsiou získanom materiáli k dispozícii málo tkaniva (Kapitola 4.2). V skorych fázach akútnej a chronickej rejekcie boli okolo krvnych ciev infiltrované bunky, ktoré boli zhodné s infiltrovanymi bunkami v dychacích cestách. Zápalová reakcia bola pocas akútnej rejekcie prudsia ako pocas chronickej rejekcie, s nápadnou prítomnostou makrofágov.

Táto stúdia ukazuje, ze zistenie specifickych buniek v biopsiou získanych vzorkách z plúc, môze umoznit viac nazriet do imunologickych procesov, ktoré prebiehajú v transplantovanych plúcach a to aj vtedy, ak je k dispozícii málo tkaniva. A co je dôlezité v rámci nálezov predchádzajúcich kapitol: Ukazuje sa skutocne, ze po plúcnej transplantácii sa odohrávajú tie isté reakcie u cloveka, aké sme zistili po plúcnej transplantácii u krysy. Takto môzu vyskum na pokusnych zvieratách a klinicky vyskum spolu umoznit hlbsie nazriet do mechanizmov, ktoré vedú ku vzniku bronchiolitis obliterans po transplantácii plúc.

5 Príciny bronchiolitis obliterans v plúcnych transplantátoch

V konklúzii prichádzame k následujúcej hypotéze o vzniku bronchiolitis obliterans po plúcnej transplantácii: chronická rejekcia vo vcasnom stádiu po transplantácii zaprícinuje nepatrné poskodenie na dychacích cestách transplantovanych plúc. Avsak dôlezitejsie je to, ze tá istá rejekcija poskodí lokálny obranny systém v transplantovanych plúcach natolko, ze tento uz nemôze normálne fungovat pri postihnutí infekciami. Ak sa potom plúca infikujú vírusom z dychacích ciest, zaprícinuje to abnormálne prudkú a dlhodobú bronchiolitis so silnym zjavením hornych ciest dychacích: obraz bronchiolitis obliterans. Ostatné faktory, ktoré tiez zaprícinujú zápalové reakcie v transplantovanych plúcach , napr. ischémia, môzu tento proces este zosilnit.

Prevencia proti bronchiolitis obliterans by sa mala uberat dvomi smermi. Po prvé agresívnym potlacovaním rejekcie vo vcasnom stádiu po transplantácii plúc tak, aby zostal nedotknuty lokálny obranny systém v plúcach. Po druhé potlácaním abnormálne prudkych zápalovych reakcií v transplantovanych plúcachpo infekcii. Zrejme dnesné liecebné metódy niesú v tomto dostacujúce. Rejekcia sa lieci vzdy

viacerymi imunopresívami, dodávané sú permanentne, vcítane corticosteroidov, ktoré sú tiez silnou brzdou zápalov. Mozno si vsak predstavit, ze tieto lieky, v súvislosti s moznostou váznych vedlajsích úcinkov, sú casto podávané neskoro a v nedostatocnych dávkach. Okrem toho môze lokálna koncentrácia but nedostatocne silá na to, aby potlacila prudkú zápalovú reakciu. Jeden spôsob ako tymto problémom predíst zrejme je, lokálne podávat lieky do transplantovanych plúc, ako sa aj liecy astmaticy bronchitis, stále viac liekov sa berie inhalacne. To sa ukazuje aj po transplantácii plúc ako dobrá alternatíva vzhladom k tomu, ze reakcie, ktoré vedú k bronchiolitis obliterans, sú vyrazne lokalizované v dychacích cestách a okolo ních, a sú teda inhalovanym látkam dobre prístupné. Rejekcia ako aj zápal by mohli byt liecené tymto spôsobom.

Iná, este experimentálna, liecba plúcneho transplantátu k predídeniu rejekcie menovite BALT je, oziarit plúca darcu este skôr, ako sa tieto darcovi odoberú, cím zmiznú silno imunogénne lymfocyty z BALT-u plúc, ktoré sa majú transplantovat. Struktúra BALT-u by v tychto oziarenych plúcach mohla zostat lepsie intaktná a dokonca by mohla umoznit repopuláciu lymfocytmi príjemcu. Mozno sa tymto môze obnovit funkcia BALT-u a vznikne normálna obrana proti infekciám.

Záver

Z horeuvedeného pojednania sa ukazuje, ze transplantácia plúc sa v súcastnosti dá lahko technicky vykonat, ale po transplantácii nie je este povedané posledné slovo. Transplantovany pacient sa este ani zdaleka nezbavil svojich problémov a uz vonkoncom nie svojich lekárov. Urcity cas sa transplantovanym plúcam darí dobre, ale potom nastanú opät nové problémy v podobe bronchiolitis obliterans. Hoci zpociatku neriesitelné, zacíname pomaly rozuzlovat klbko prícin, spôsobujúcich toto zradné ochorenie. Tym môzu vzniknút racionálne terapie k prevencii a v prípade potreby k liecbe bronchiolitis obliterans v transplantovanych plúcach.

Met dank aan

Wie iets vindt, heeft slecht gezocht
Rutger Kopland

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Jobst